INTERROGATION OF *PITX3* FOR BLASTOCYST COMPLEMENTATION IN THE GENERATION OF LENS

THESIS SUBMITTED TO THE UNIVERSITY OF MINNESOTA GRADUATE SCHOOL

BY

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE DEGREE IN STEM CELL BIOLOGY

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DECEMBER 2015

ACKNOWLEDGEMENTS

I would like to thank Dr. Walter C Low, for providing me with the opportunity to work in the lab and for all the guidance. It has been a pleasure to work in the lab and learn such a great deal. I would like to thank, Holly Hewitt for answering all of my questions, and for helping out in the sectioning the eyes. I would like to thank Zach Schultz for assisting in the cutting and staining of the eyes and all the lab members for all their help and guidance.

I take this opportunity to also thank, Sarah Weatherman (UIC, UMN), Mark Sanders (UIC, UMN) and the Recombinetics team.

I extend my gratitude to Dr. Susan Keirstead for the support and guidance. Finally, I wish to thank the University of Minnesota, Stem cell institute, Master's program for providing me with the opportunity to learn.

ABSTRACT

Pituitary homeobox 3 (Pitx3) is a transcription factor that is involved with ocular development. Knockout of the *Pitx3* gene in mice results in defects in the lens. We have developed *Pitx3* knockout in porcines as a model system to assess the effects of blastocyst complements with human umbilical cord blood stem cells (hUCBSC) and human induced pluripotent stem cells (hiPSC). TALENS were used to knockout (KO) *Pitx3* in porcine fibroblasts. DNA from KO fibroblasts were transferred to enucleated porcine oocytes to generate KO blastocysts. KO blastocysts and KO blastocysts with injections of hUCBSC or hiPSCs were transferred to surrogate pigs and allowed to develop to 62 days in gestation for comparisons with wild type (WT) porcine fetuses at the same gestational age. WT fetuses exhibited well-formed lenses. In contrast, the lenses in *Pitx3* KO fetuses were mal-formed and poorly developed. Complementation with hiPSCs or hUCBSC did not rescue the KO lens phenotype. These results demonstrate that *Pitx3* KO in porcine results in arrested lens development similar to that observed in mice. Lens complementation with hiPSC and hUCBSC, however, was not successful, possibly due to poor contribution of "non-ground state" or 'primed' human stem cells to the developing porcine fetus.

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INTRODUCTION

In the past decade the field of stem cell biology and regenerative medicine has grown with leaps and bounds. The extensive capacity of self-renewal and potency of pluripotent stem cells has led to various cell replacement therapies, tissue engineering and regenerative medicine. Current strategies of organ transplantation are limited by problems such as lack of donors, HLA type matching, immune rejection and graft versus host disease, etc. (Choumerianou et al., 2008). Thus, new strategies for the development of donor-derived organs are underway and stem cells contribute greatly as therapeutic tools against many degenerative diseases, not limited to, Parkinson's Disease (PD), Alzheimer's Disease (AD), stroke, Huntington's disease, muscular dystrophy, and anterior segment macular dysgenesis (ASMD) (Hirai, 2002; Lindvall et al., 2004; Lunn et al., 2011). Incorporating the technology of blastocyst complementation and using chimeras, I aimed to create human donor-derived cells in porcine blastocysts, by manipulating a single gene of interest.

1.1 Blastocyst complementation to Generate Organs and Cells

Blastocyst complementation was first developed by Chen et al. 1993, to evaluate *RAG-2* gene, whose deficiency causes the failure of B and T cell maturation. Knock out (KO) models for this system failed since it is embryonically lethal. But when they, injected normal or modified embryonic cells (ES) into a *RAG-2* KO blastocyst, it lead to the development of chimeras with mature B and T cells, contributed by the ES cells. The growth of these chimeric embryos can be arrested at any stage and gene expression can be studied (Chen et al., 1993). Similarly, based on the experiments performed by Chen et al. the lens complementation system was developed. This system used the previously

mentioned aphakia mouse line, which fails to generate completely developed ocular lenses (Semina et al., 1998). Normal mouse ES cells were injected into mutant mouse blastocysts, and allowed to develop in a surrogate. These chimeric animals were able to generate lens, whose eyes responded to visual stimulus and were free of cataracts or other irregularities (Liégeois et al., 1996).

1.1.1 Inter-specific mouse-rat chimeras

The next step to using this technology was to try and create donor-derived organs in the recipient organism. Kobayashi et al. used a gene KO blastocyst complementation model with iPSCs or ESCs as the donor cells (Kobayashi et al., 2010). Induced pluripotent stem cells (iPSCs), were first developed by Takahashi and Yamanaka in 2006, by taking mouse fibroblasts and reprogramming them into pluripotent cells, similar to ESCs, by the overexpression of Oct4, Sox2, klf4 and c-Myc (OSKM) transcription factors via viral vectors (Takahashi and Yamanaka, 2006). Since then various adult human cells have been reprogrammed into iPSCs (Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007). In the 2010 paper by Kobayashi et al., they used a homozygous *Pdx1*deficient blastocyst. *Pdx1* gene is necessary for pancreatic development. With the gene disrupted it created a niche, where mouse iPSCs or ESCs with the working *Pdx1* gene took its place. The blastocysts were then transplanted into a surrogate, with the resulting chimeric pups having a donor-derived pancreas. After success of the experiment, an attempt was made for inter-specific complementation. Thus, they used rat iPSCs or ESCs and injected them into *Pdx1* knock-out mouse blastocyst, and discovered that the resulting chimera possessed completely rat (donor-derived) pancreas, refer figure 1 (Kobayashi et al., 2010). Similarly, by using *Sall1* KO mouse blastocyst, donor-derived kidneys were generated using mouse

iPSCs. *Sall1* gene is critical for nephrogenesis and the gene disruption created another niche. When iPSCs with the working gene were injected, they occupied the niche thus, creating donor-derived kidneys (Usui et al., 2012). But, mice and rats are evolutionarily close and cannot be used for studies in humans. Thus, a scale-up was needed to have large enough organs to be used for human transplantation, if possible.

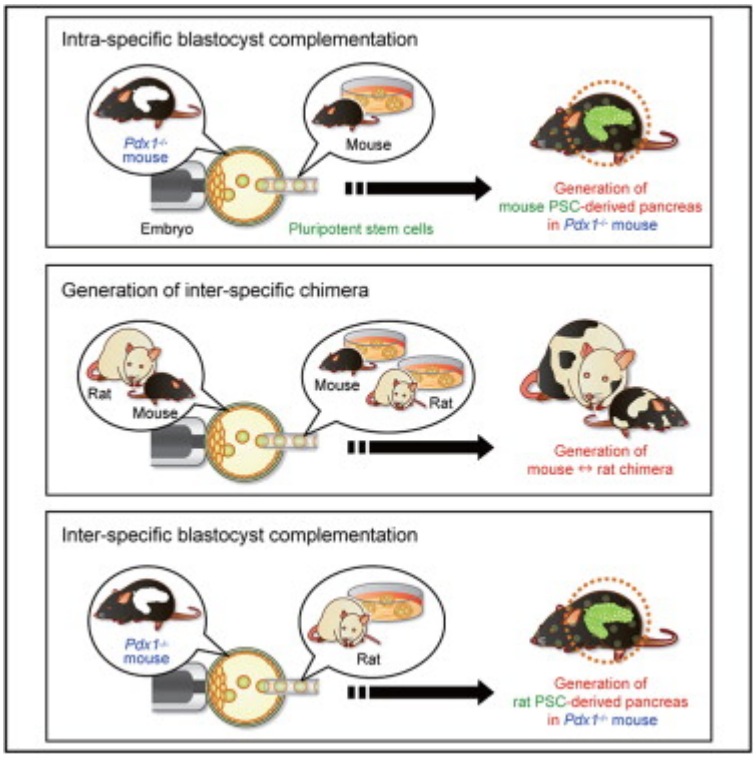

FIGURE 1: The top panel depicts intra-specific complementation, where the resulting chimeric pups develops pancreas generated from donor PSCs from using a *Pdx*$^{-/-}$ blastocyst. The middle panel portrays inter-specific generation of chimera between a rat and mouse, with the mosaicism depicted in the resulting pup. The last panel shows inter-specific blastocyst complementation, where they generated rat pancreas in a mouse, by using rat PSCs and injecting into *Pdx*$^{-/-}$ mouse blastocyst (Kobayashi et al., 2010).

1.1.2 Blastocyst complementation using porcine blastocysts

To generate human sized organ for transplantation, researchers began to test if pigs were a feasible model system. Recently, it was demonstrated that blastocyst complementation could be performed using porcine blastocysts. *Hes1* gene, which is required for biliary system development, was overexpressed under the *Pdx1* promoter to inhibit pancreatic development in pigs. The *Pdx1-Hes1* transgene was introduced into porcine oocytes by a process called "sperm injection mediated gene transfer" (Kurome et al., 2006) and the resulting fetuses were checked for disabled pancreatogenesis. To make things easier, fibroblasts from these pancreatogenesis disabled pigs were used as the donor nucleus, to perform SCNT-somatic cell nuclear transfer and generate pig clones with the *Pdx1-Hex1* transgene. SCNT is the process of injecting donor nuclei into a recipient oocyte to generate an offspring that is genetically identical to the donor (Campbell et al., 1996; Polejaeva et al., 2000). Thus, clone transgenic pigs with disabled pancreatogenesis were generated. The blastocysts from such male clones was used for blastocyst complementation. At the morula stage, it was complemented with blastomeres from a WT female pig embryo, cloned to express a fluorescent protein. This was then transferred to a surrogate and the fetuses were analyzed and the generation of donor-derived pancreas using porcine blastocysts was reported. A schematic representation of the procedure is depicted below in figure 2 (Matsunari et al., 2013).

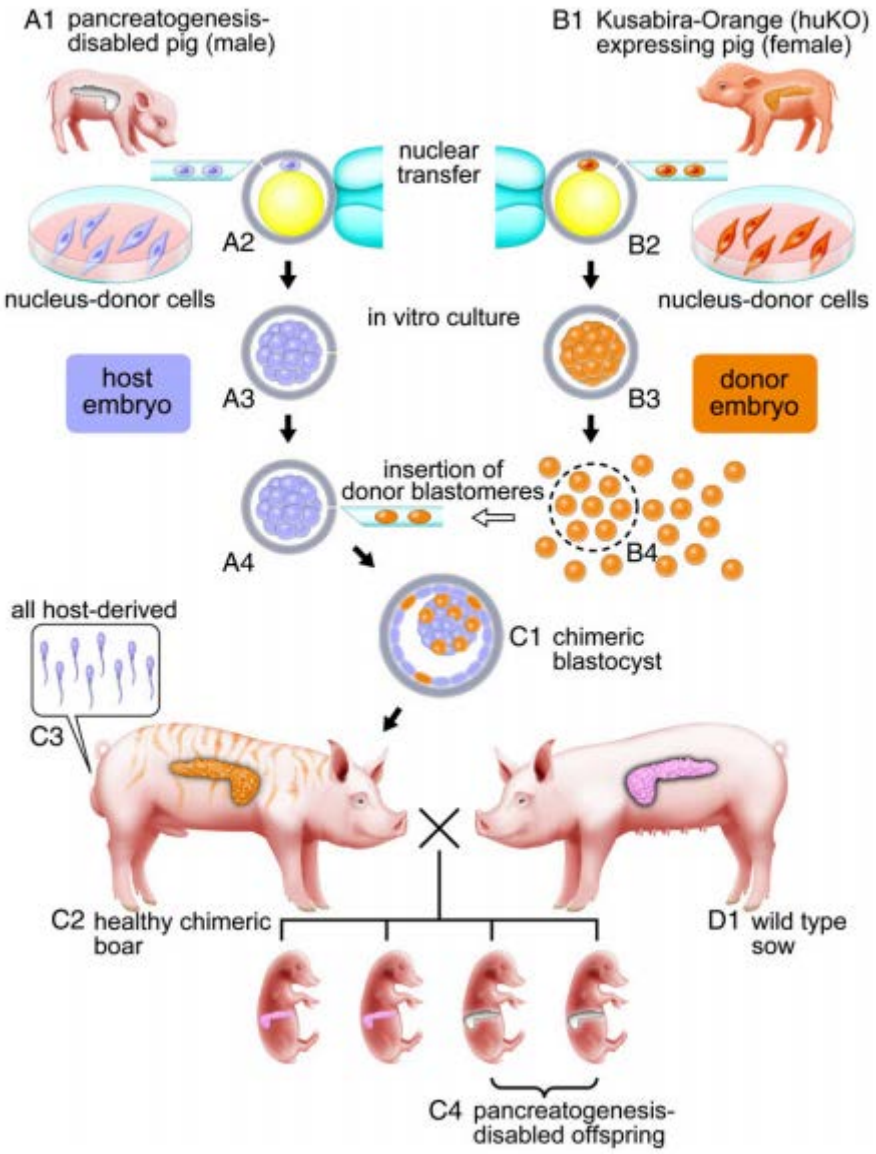

FIGURE 2: Schematic of generation of donor-derived pancreas using porcine blastocysts for complementation. Female WT porcine blastomeres with fluorescent dye were injected into male Pdx1-Hes1 disabled blastocyst, thus producing male offspring with a fully functional pancreas (Matsunari et al., 2013).

1.2 Roles of *PITX3* gene

Pituitary homeobox 3 protein, which is encoded by *PITX3* gene, is a transcription factor belonging to the PITX3/REIG homeobox gene family (Semina et al., 1998). In mice, the *Pitx3* cDNA was isolated by using a probe containing Pitx2, and three different clones were identified. Since *Pitx1* and *2* had already been discovered, and based on the homology of the third clone to the other two it was designated as *Pitx3*. Studies in mice have informed us that *Pitx3* expression starts from E 10 (embryo day 10) in the lens placode and lens pit. At E 12, expression was observed in the midbrain, head muscle, tongue, sternum, and incisor primordia (Semina et al., 1997, 1998). *Pitx3* was mapped to the mouse chromosome 19 and is situated at the aphakia region, suggesting its involvement in the diseased condition. Aphakia is a recessive mutation, where aphakia homozygous mice are characterized by lack of lens, and closed eyelids, while heterozygous mice depict a normal phenotype (Semina et al., 1997). Reiger et al., in 2001 reported not one but two deletions in *Pitx3* gene as the cause for the aphakia phenotype, figure 3. These deletions include one major 1423 base pair (bp) deletion that removed exon 1 and the adjacent intron 1, as well as the promoter sequence, and a minor 642 bp deletion upstream of the major deletion that removed the transcription start site thus, disrupting the transcription of *Pitx3* (Rieger et al., 2001). *Ptx3* is the rat homolog of *Pitx3* (mouse). It was found to be important in the development of the mesencephalic dopaminergic system (mesDA or DA). Similar to the rat homolog, the human *PITX3* gene was mapped to chromosome 10q25. The *PITX3* (human) gene exhibits an 88% homology with *Pitx3* (mouse) gene at the nucleotide level, and a 99% homology at the protein level. *PITX3* also functions in eye development, in addition to the development of the DA system (Semina et al., 1998).

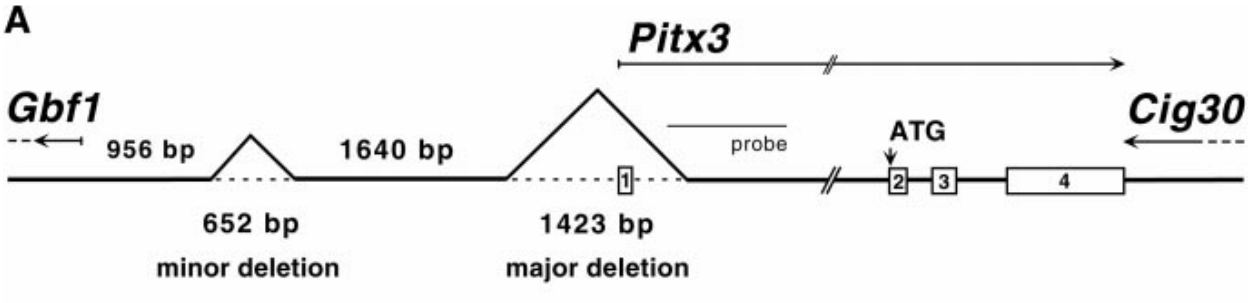

FIGURE 3: Diagram indicates the double- deletion of *Pitx3* gene in aphakia mice. The horizontal arrows depict the transcriptional units. Exons are indicated by the numbered boxes. The 625 bp minor deletion and the 1423 bp major deletion are separated by a wildtype sequence, 1640 bp in length. (Rieger et al., 2001)

1.2.1 Function of *Pitx3* in the development of the DA system and importance in Parkinson's disease

The midbrain dopaminergic (DA) system is responsible for controlling various functions such as reward, cognition, emotional behavior and voluntary movement. The degeneration of these cells has been shown to result in PD, while the misregulation in the signaling of dopamine neurotransmitter is involved in various behavioral disorders and schizophrenia (Damier et al., 1999; Domburg and Donkelaar, 1991; Schultz, 2001; Seeman et al., 1993). There are 3 groups of DA neurons, and most reside in the ventral tegmental area (VTA) and substantia nigra (SN), while others are found in the retrorubral field (RRF). These together, comprise the mesencephalic system. The DA neurons in the SN project and innervate the striatum, especially the caudate and putamen and, control voluntary movements. In PD, preferential degradation of these nigrostriatal neurons has been observed (Domburg and Donkelaar, 1991; Lindvall et al., 1984).

Many transcription factors have been associated with the development of DA neurons such as *Lmx1b, Nurr1* and *Pitx3*. They are expressed in the dorsal midbrain to a greater extent and partake in neuron migration and maturation (Nunes et al., 2003; Smidt et al., 2004a). Other transcription factors include fibroblast growth factor 8 (FGF8), sonic hedgehog (Shh) and engrailed 1 and 2. Shh along with FGF8 is important early in the development of the brain from the neural plate and commit the cells to a particular part of the midbrain from which dopaminergic neurons later arise. Shh is later expressed in the lateral part of the midbrain to a greater extent, than in the medial part, which may lead to the formation of subtypes of dopamine neurons (Hynes and Rosenthal, 1999; Hynes et al., 1995a, 1995b; Lin and Rosenthal, 2003; Wallén et al., 1999). Nurr1 orphan nuclear hormone, has been proven essential for the maturation of the mesencephalic DA neurons. It has been shown that, *Nurr1* knock out (KO) mice do not express the enzyme tyrosine hydroxylase (TH), an essential component in the biosynthesis of dopamine neurotransmitter (Baffi et al., 1999; Castillo et al., 1998; Le et al., 1999; Saucedo-Cardenas et al., 1998; Wallén et al., 1999; Witta et al., 2000; Zetterström et al., 1997, 1997). Lmx1b; LIM homeodomain transcription factor, is required for the specification of mesencephalic DA neuron progenitors (Smidt et al., 2000). *Pitx3* expression is now required for further development, and the gene falls downstream of the transcription factors stated above.

As previously mentioned, *Pitx3* has been reported to be a particularly important transcription factor in nigrostriatal DA neuron development. *Pitx3* expression has been reported in the developing eye and striatum by E 11 and continues throughout adulthood in mice (Gage et al., 1999; Smidt et al., 1997). Previous studies have shown that *Pitx3* expression is localized specifically in the SN and VTA, but is important for the

maintenance of just the SN neurons. Mutation studies in aphakia mice has shown an absence of TH in the SN, especially the caudate and putamen, while leaving the VTA undisturbed (Hwang et al., 2003; Nunes et al., 2003). Studies have also shown the requirement of *Pitx3,* for the terminal differentiation of neurons in the mes-DA system (Smidt et al., 2004b). In the mouse midbrain TH, is expressed at E11, right after *Nurr1* which is expressed at E10 and around the same time as *Pitx3* expression (Maxwell et al., 2005). It was observed that the SN and VTA differ in their requirement of *Pitx3*, since two different groups of DA neurons were discovered. The VTA, which originates from the medial neuroepithelium, first expresses TH before expressing Pitx3, thus it does not require *Pitx3* for TH activation. On the other hand, the SN arising from the ventrolateral part of the neuroepithelium, first expresses *Pitx3* and those cells then expresses TH, which gives rise to mature dopaminergic neurons. Thus, *Pitx3* is essential for the maturation and maintenance of these neurons in the midbrain (Kawano et al., 1995; Maxwell et al., 2005; Smidt et al., 2004b). It has since been reported that these SN DA neurons have a high susceptibility of degeneration in Parkinson's disease. (Barzilai and Melamed, 2003).

1.2.2 Function of *Pitx3* in ocular development

The development of the eye has been examined in various animal models including zebrafish (Shi et al., 2005), xenopus (Grainger, 1992; Henry and Grainger, 1987; Khosrowshahian et al., 2005; Ogino et al., 2012), chicken and mouse (Mochizuki and Masai, 2014; Ogino et al., 2012).

The eye embryonic development has been used as a cell differentiation model and has been thoroughly investigated to delineate its various stages (McAvoy et al., 1999). After

gastrulation, the lens develops from the pre-placodal ectoderm (PPE), assisted by various transcription factors. The different stages of vertebrate lens development along with the major signaling factors, are depicted in figure 4 (A-G). The cells around the anterior neural plate begin to express certain pre-placodal genes like, *Hes4* (Murato and Hashimoto, 2009), *Dlx- 3, 5, 6* (Akimenko et al., 1994; Bhattacharyya et al., 2004; Feledy et al., 1999; Luo et al., 2001; Quint et al., 2000; Yang et al., 1998) to name a few, refer figure 4H. The presumptive lens ectoderm or (PLE) is included in the PPE and, in the next stage of development the PLE genes such as, *Pax6*; which is required for lens induction (Grindley et al., 1995; Li et al., 1994; Nornes et al., 1998; Walther and Gruss, 1991; Zygar et al., 1998) as well as, *Six3* (Bovolenta et al., 1998; Kobayashi et al., 1998; Oliver et al., 1995; Zhou et al., 2000), begin to be expressed. *Pax6* mutations present a 'small eye' phenotype in rats and mice, while in humans it is responsible for the absence of an iris (Quiring et al., 1994). All the above mentioned genes facilitate 'lens specification'. Eventually, the PLE makes contact with the optic vesicle, which is formed by the protrusion of the presumptive retinal region, around the same time. Once this initial contact has taken place, the cells of the PLE start to elongate to give rise to the lens placode. Differentiation genes such as, *FoxE3* (Blixt et al., 2000; Shi et al., 2006), *Mab21l1* (Yamada et al., 2003) and importantly, *Pitx3* are involved in this process (Pommereit et al., 2001; Semina et al., 2000; Zilinski et al., 2005).

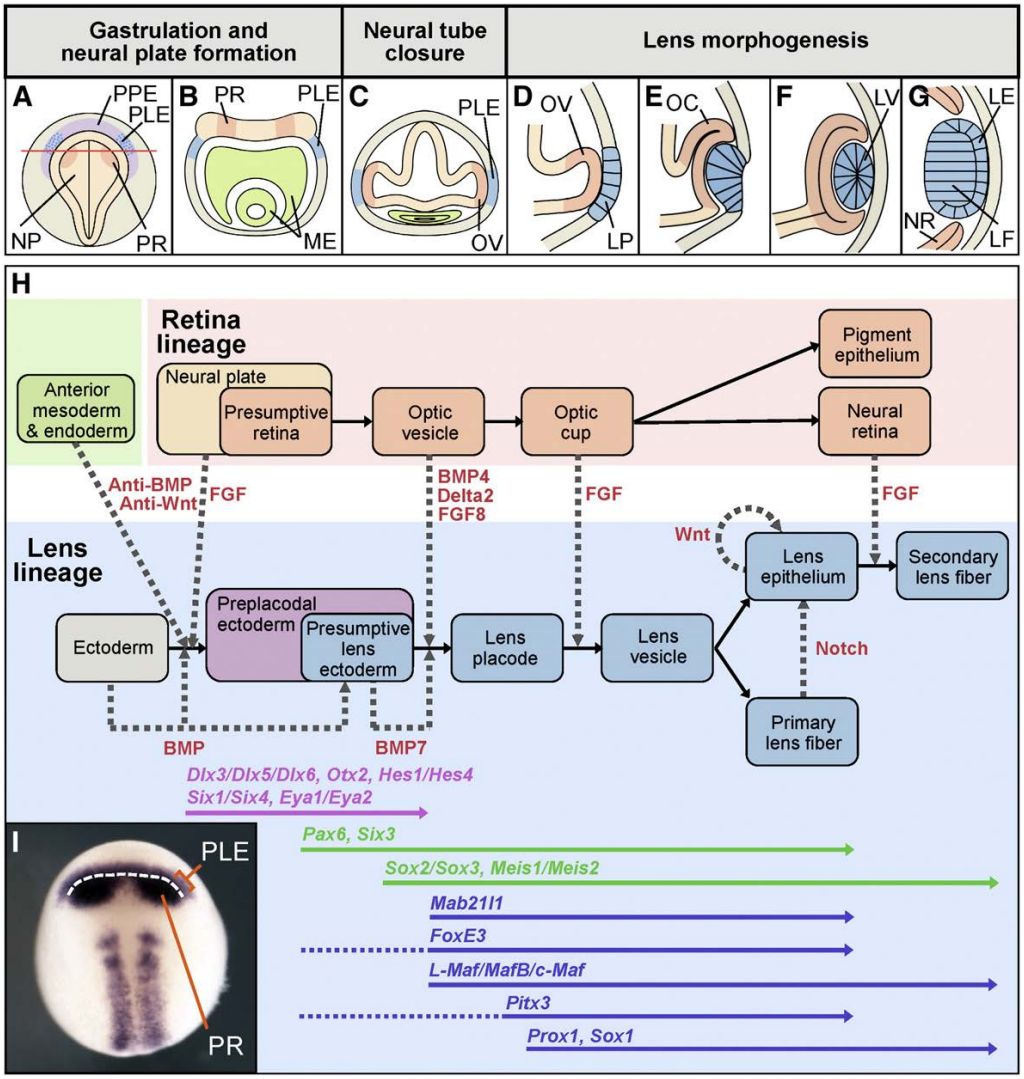

FIGURE 4: The above figure depicts the different stages of vertebral lens and retinal development. **A- G** is a pictorial representation of different stages of development of the eye in xenopus after gastrulation. **H** represents the different stages of lens and retinal development in xenopus, along with the various important signaling factors such as Wnt, BMP, FGF and Notch. While below it are various transcription factors, that are required at different time points during development. The length and placement of the arrows depict when they start expression, continuing through the stage they are expressed. **I** is an in-situ hybridization analysis of Pax6, in a xenopus embryo, with the presumptive lens and retina represented (PLE and PR, respectively). (Ogino et al., 2012)

The elongated cells in the lens placode begin to differentiate and form primary lens fibers, where cell cycle is arrested, the cells become enucleated and lose many other organelles

such as mitochondria and golgi. Due to an upregulation of crystallins such as beta and delta crystallins, the cells get crystallized (Mcavoy, 1980; Piatigorsky, 1981). At the anterior portion of the lens, a single layer of epithelial cells exist, that retain their nucleus and membrane bound organelles, remain in the cell cycle. These epithelial cells, surrounding the lens fiber cells, proliferate and differentiate into fiber cells when required and *Pitx3* is said to be essential specifically for this differentiation (Ogino et al., 2012; Shi et al., 2005).

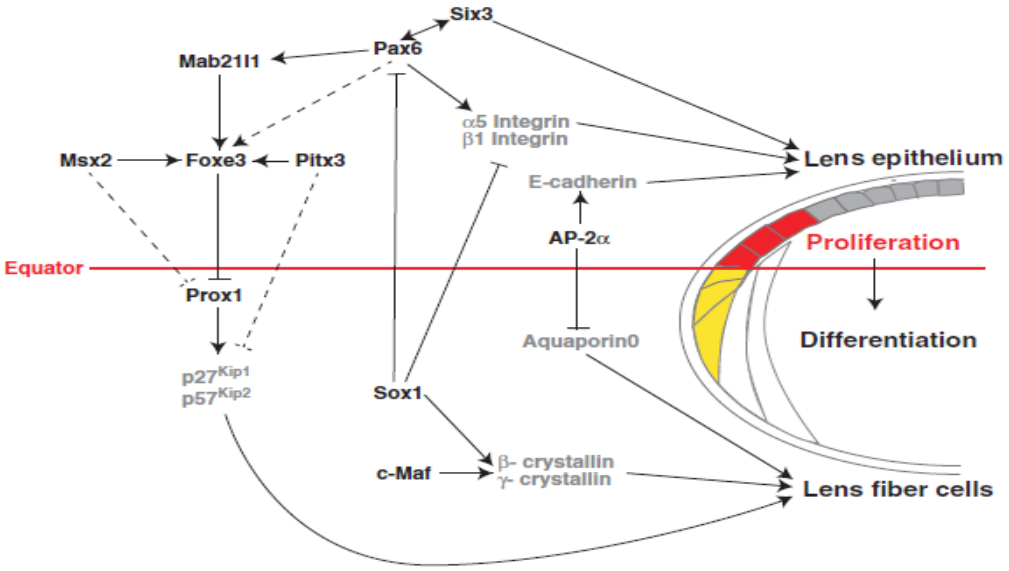

FIGURE 5: Major transcription factors involved in regulating the ocular lens development. Depicts *Pitx3* action on lens, downstream of *Pax6* and *Six3*, major lens induction genes, as well as, *FoxE3* another major differentiation gene (Mochizuki and Masai, 2014).

Pitx3 expression in mice, can first be observed during lens placode formation, figure 4H (Medina-Martinez et al., 2009; Pommereit et al., 2001). As previously stated by Semina et al., 1998 and 2000, the aphakia condition is caused by a natural mutation of the *Pitx3* gene in mouse. This deficiency in gene expression leads to failure in the development of the lens epithelium and lens fibers from the lens vesicle, which eventually leads to the degeneration

of the primary lens fiber cells (Medina-Martinez et al., 2009; Rieger et al., 2001). Thus, *Pitx3* is required for lens epithelial cell population maintenance, and indirectly influences the maintenance of lens fibers, formed by the differentiation of lens epithelial cells (Mochizuki and Masai, 2014; Ogino et al., 2012). Pitx3 protein also plays a crucial role in retinal cell maintenance. Loss of differentiated retinal neurons or cell death, with significant reduction of certain retinal cell types has been reported in *Pitx3* mutated animals, providing evidence of its involvement in retinal development and maintenance (Khosrowshahian et al., 2005; Shi et al., 2005). Aphakic mice also present with blindness due to absence of lens, pupil and iris, a condition called micropthalmia. The eyelids are closed, with retinal folds invading the vitreous chamber. Due to the malformation of the anterior chamber, the lens retains its connection to the cornea, and at the lens stalk stage, development is arrested (Varnum and Stevens, 1968). Finally, mutations in the human *PITX3* gene have been observed in many autosomal dominant cataracts, as well as, ASMD (Semina et al., 1998). Many cell replacement and transplantation therapies are under investigation. But, in order to scale these studies to a human level, larger animal models that better mimic human conditions are required. Porcine models would be a step ahead of mouse or rat models. Humanized mouse models have been widely used to model various diseases, but unfortunately, they do not mimic the diseased conditions as they present themselves in humans (Lunney, 2007). The technology of blastocyst complementation maybe the next step, by providing a way to analyze human genes and human cells at any stage of development.

1.3 Gene editing with TALENs

Evaluation of gene function is possible by various powerful methods of targeted gene inactivation, such as via homologous recombination (HR). Nevertheless, several limitations such as low efficiency of correct insertions into the target genome, labor-intensive and time-consuming selection and screening, and potential adverse mutagenesis (Capecchi, 2005). New approaches of genome editing, such as transcription activator like nuclease (TALENs), zinc finger nucleases (ZFN) and CRISPER-Cas9, have emerged in the past decade and have enabled researchers to manipulate a variety of genes in various organisms and cells. The basis of this technology is the use of engineered nucleases that contain particular DNA binding domains and are fused to various DNA cleavage units, which has enabled precise and efficient genetic modifications by the induction of double stranded breaks at targeted regions on the DNA strand. The double stranded breaks are then repaired by homology directed repair and non-homologous end joining (NHEJ) (Carroll, 2011; Urnov et al., 2010; Wyman and Kanaar, 2006). Transcription activator-like effectors (TALE) are specific DNA binding proteins. They were fused to the Folk1 nuclease catalytic domain, which created double stranded breaks. In cells, double stranded breaks are usually repaired by NHEJ and usually causes insertions or deletions. Thus, this strategy was used to delete or disrupt a *Pitx3*, the gene of interest for our experiments (Cermak et al., 2011; Christian et al., 2010).

1.4 Complementation of porcine parthenogenic Blastocysts using human pluripotent cells

In our current research we aimed to interrogate the role of *PITX3* gene during development, and investigate if human dopaminergic neurons and lens could be developed using blastocyst complementation. Previous work has established that complementation studies in porcine blastocysts using human stem cells is in-fact possible (Swaminathan. P., 2014). We started by investigating the integration of different human pluripotent stem cells such as, human iPSCs (hiPSCs), umbilical cord blood stem cells (UCBSCs) and human multipotent adult progenitor cells (hMAPCs) (Nan et al., 2005; Xiao et al., 2005), into the inner cell mass (ICM) of porcine parthenogenic blastocysts. In order to visualize and quantify the integration of human cells, immunohistochemistry (IHC) was performed using the blastocysts, they were labeled with an antibody against human nuclear antigen (HNA). Parthenogenic zygotes at Day 0 were generated by electrical activation and injected with approximately 10 human stem cells. They were then cultured until they reached the blastocyst stage (6 days after injection) and fixed and stained for HNA on day 7.

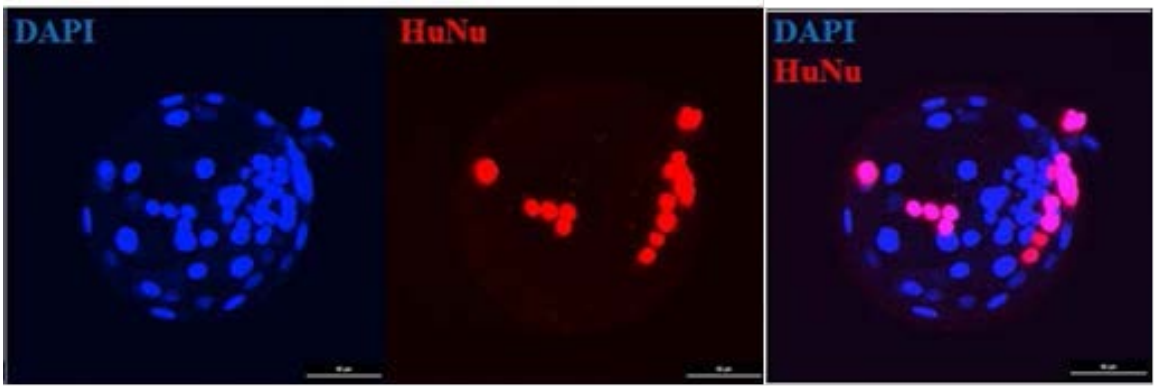

FIGURE 6: Immunohistochemistry of day 7 porcine parthenogenic blastocyst injected with UCBSCs and labeled with anti-human nuclear antigen or HNA (HuNu). **A** shows all

the cells labeled with DAPI (blue). **B** indicates the presence of human cells that stained positive for HNA (red). While panel **C** is a merge. Scale: 50μm (Swaminathan. P., 2014).

The number of cells integrated into the ICM was then quantified, after which the blastocysts containing human pluripotent cells were transferred to a surrogate and allowed to remain 28 days in gestation. They were then excised, and fixed, embedded and sectioned. The sectioned tissues were stained for HNA, to visualize the contribution of human cells in the organs. (Swaminathan. P., 2014).

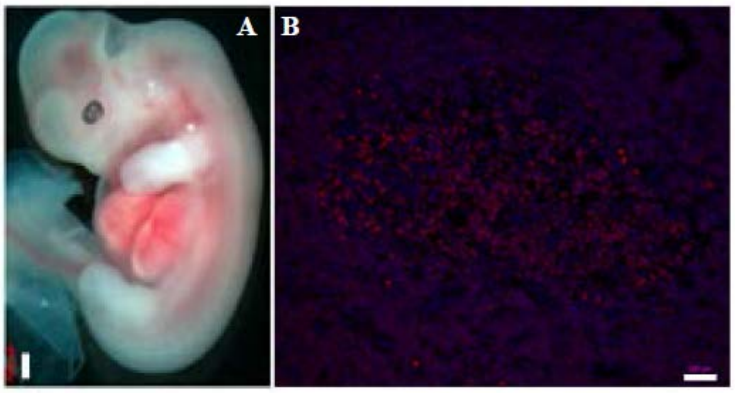

FIGURE 7: Identification of human cells in embryos generated via blastocyst complementation of porcine parthenotes. **A** depicts a 28 day old parthenote. **B** is a florescent image, of a section of liver tissue from the parthenote, stained with anti-Human nuclear antigen (HNA). Red= HNA; Blue= DAPI. Scale, **A**: 1000μm and **B**: 100μm (Swaminathan. P., 2014).

1.5 Generation of human-porcine chimera using *PITX3* KO blastocysts for complementation with hiPSCs and UCBSCs.

Based on the success of generating human-porcine pathenogenic embryos with contribution of the human stem cells in organ development, the next step was to create a niche for these human cells to specifically integrate. The study was designed similar to the ones by Matsunari et al, and Kobayashi et al., as previously mentioned. But for our studies to generate a *PITX3* KO we used Transcription Activator-like Effector Nucleases or, TALENs for gene editing. Recently, Carlson et al. demonstrated the efficiency of TALENs in generating knock outs in porcine fibroblasts. A TALEN pair with an EGFP reporter, used as indicator, was injected into zygotes to confirm integration. A pair of gene specific TALEN mRNAs were injected to target specific genes and produced insertions or deletions, thus generating KO in porcine fibroblasts. Clones were created using chromatin transfer (Carlson et al., 2012).

For the purpose of our system, TALEN mRNAs specifically targeting genes *Nurr1*, *PITX3* and *Lmx1a* were used. PCR analysis confirmed the disruption of the genes, thus successfully generating porcine KO fibroblasts, shown in figure 8 (Carlson et al., 2012, Swaminathan . P., 2014) *Pitx3* KO porcine embryos were then generated by performing SCNT using chromatin from *Pitx3*$^{-/-}$ fibroblasts and injecting into porcine oocytes. These blastocysts were transferred into a surrogate sow and allowed to remain 62 days in gestation thus, generating *Pitx3* KO porcine embryos. Bi-allelic KO was confirmed on analyzing tissue sample from each embryo (Swaminathan. P., 2014).

Similarly we used the previously generated *Pitx3* KO morulae and performed blastocyst complementation studies by injecting human iPSCs and UCBSCs. These were cultured until they reached the blastocyst stage and were then transferred to a surrogate sow for development up to 62 days in gestation.

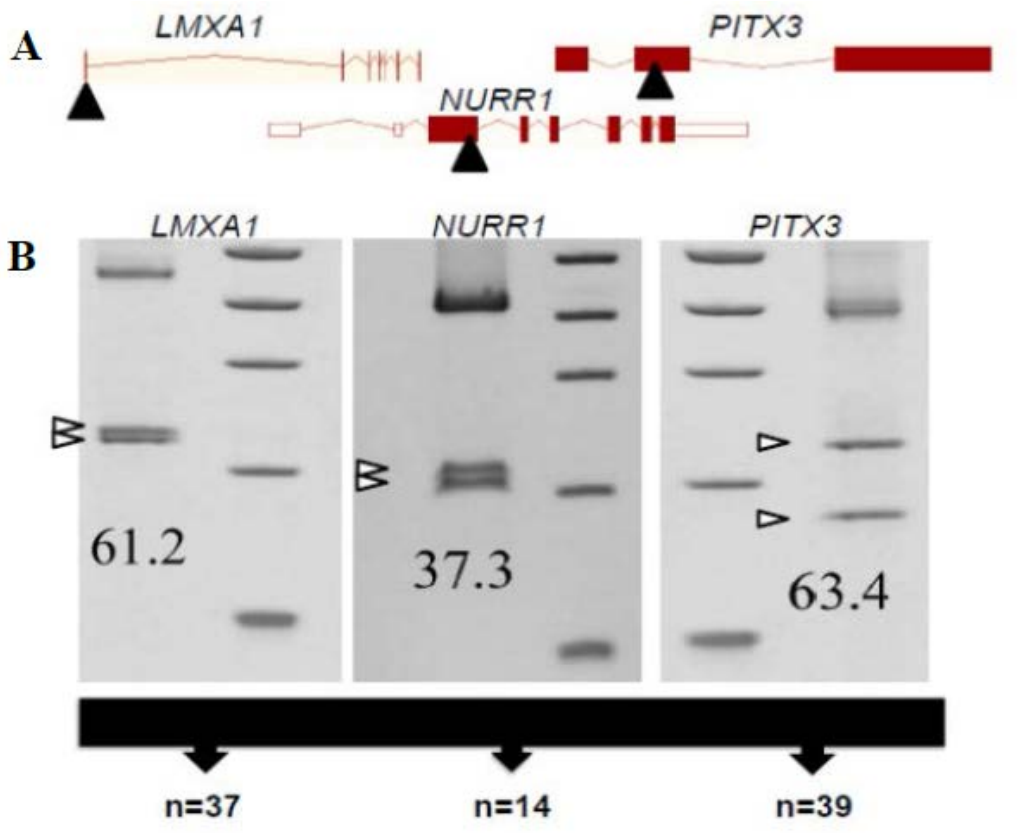

FIGURE 8: Gene knock-outs in porcine fibroblasts mediated by TALENs. **A** depicts the TALEN constructs with specific cleavage sites for *Lmx1a*, *Nurr1*, and *Pitx3* genes. **B** is the PCR analysis of porcine fibroblasts to confirm the cleavage of the genes after TALEN mRNA targeting. The cleavage products are indicated by double arrows (Swaminathan. P., 2014).

Thus, we generated 3 groups of embryos: *Pitx3* KO porcine embryos and the 2 chimeric groups, *Pitx3* KO blastocysts complemented with hiPSCs and *Pitx3* KO blastocysts

complemented with UCBSCs. Wild type (WT) porcine embryos, whose development was arrested at 62 days in gestation were used a controls. In this particular report, the focus will be on the contribution of *PITX3* in the generation of a normal lens in the KO embryos. The eyes from the porcine embryos were excised, fixed and embedded. The tissue was then sectioned for analysis. Morphology of the eyes was assessed with histological analysis and IHC was performed on the tissues using and antibody against HNA to detect the presence of human cells. Although, we were not able to detect the presence of any human cells in the lens or retina, we were however able to see certain differences in retinal morphology in some of the chimeric embryos complemented with UCBSCs. Here we report a method of successfully generating a human-porcine chimera, which has been highly valuable in assessing the significance of *Pitx3* for the normal embryonic development of the eye.

METHODS AND MATERIALS

TALEN DESIGN

The knockouts for the interrogation of *PITX3* gene were generated through somatic cell nuclear transfer (SCNT) as described previously by Carlson et al, 2012. A brief description of TALEN design is as follows, 'TALE-NT' software was used to design the TALENs (Cermak et al., 2011). Each TALEN pair that was compatible for Golden Gate cloning had intermediate arrays, which were cloned into vectors: RCIscript-GoldyTALEN, pC-GoldyTALEN, RCIscript-+231-TAL and Pc-+231-TAL. In a 20µl digestion-ligation reaction that consisted of T4 DNA ligase (50 units, New England Biolabs), Esp31 (10 units, Fermentas) and diluted in 1X ligase buffer (New England Biolabs), 150 ng of each of the plasmids along with the desired backbones were added with the arrays to join them into the vectors. The reaction mixture was then incubated in a thermocycler for 10 cycles, 5 minutes each at 37°C and another 10 minutes at 16°C. It was then heated for 5 minutes to 50°C and another 5 minutes to 80°C. About 2µl of the reaction mixture was transformed into *E.Coli* and plated on LB plates with antibiotics (Carlson et al., 2012). The TALENs were then transfected into cultured fibroblasts to produce clones. The fibroblasts were cultured in DMEM containing FBS, and the transfections were carried out using Fibroblast nucleofection kit (Lonza/ Amxa Biosystems). The transfected cells were plated and clones collected and the KO was confirmed by PCR analyses. Porcine oocytes were obtained and cultured following the protocol mentioned in Carlson et al., 2012. The DNA from these knockout fibroblasts were then transferred to an enucleated porcine ovum to create knock out blastocysts (Carlson et al., 2012).

1. BLASTOCYST INJECTIONS

As previously mentioned, *Pitx3* gene was knocked out from porcine fibroblasts via gene editing using TALENs, and KO blastocysts were generated (Carlson et al., 2012). Some of the *Pitx3* KO morulae at day 4 were microinjected with human stem cells to create chimeras. Specifically about 46 day 4 *Pitx3* KO morulae were microinjected with 7-10 human iPSCs, another set of 72 day 4 *Pitx3* KO morulae were microinjected with about 10 UCBSCs and as a control for the study, un-injected knock out porcine blastocysts (KO) and wild type (WT) porcine blastocysts were used. These were then transferred into a surrogate sow, to develop for 62 days. For the complete protocol regarding preparation of donor cells for cloning, refer to Rodriguez-Osorio et al., 2009 and Swaminathan P, 2014.

Recipient sows, about 280-320 lbs were administered with 15mg of Matrix (Altrenogest, Merck) which was mixed into the feed for about 14-17 days to prepare them to serve as surrogates. To induce superovulation 1,500 IU of PG600 (Intervert) was administered intramuscularly 24 hours after the last Matrix feeding. 82 hours later 750 units of hCG (Intervet) was administered by intramuscular injections. About 120 hours post hCG embryos were transferred into the sows for development.

2. FETAL HARVEST AND TISSUE PROCESSING

The fetuses were kept 62 days in gestation, which is a little over half way point in pigs, since *Pitx3* gene expression is strong around E 12.5, a little over half way through the gestation period in mice (Semina et al., 1998, 2000). After 62 days, the fetuses were removed. Two groups of human-porcine chimeric fetuses were obtained for, hiPSCs; 4 fetuses and UCBSCs; 8 fetuses, along with 13 KO and 16 WT fetuses as controls. Once

the fetuses were excised, a sample from each was taken for genotyping to confirm the efficient knockout of the *Pitx3* gene. Fetuses were perfused with PBS, prior to perfusing with 4% PFA. The organs were then dissected out and fixed by immersing in 4% PFA overnight and the following day were transferred to 15% sucrose. Tissues were left in sucrose for about 12 hours or until the tissue sank to the bottom, completing the fixation process. They were then embedded in OTC.

EYES: The eyes were processedas described above. They were excised and fixed with 4% PFA. They were then immersed in 15% sucrose and left overnight. The following day they were embedded in OTC, using dry ice to freeze them and stored at -80°C. Human fetal eyes were obtained commercially from Advanced Biomedical Research Inc., New Jersey and processed in the same manner.

CRYOSECTIONING: The tissue blocks were then cut into 8μm sections using a cryostat and transferred onto slides. They were air dried, preferably overnight, to ensure that the tissue adheres to the slides better. They were then stored at -20°C. Both right and left eyes of all the chimeras were sectioned and mounted onto slides, while only the right eyes were selected for sectioning for WT and KO porcine eyes, for the sake of convenience.

3. PHENOTYPIC ANALYSIS

Once the fetuses were removed after 62 days, the first step was to perform a phenotypic analysis of the eye-lids. It was previously reported that the *Pitx3* null animals exhibit closure of eyelid, or present with opaque eyelids (Rieger et al., 2001). The 4 groups of porcine fetuses hiPSC, hUCBSC, KO and WT were excised and the different organs were dissected out. The eyes were photographed before excision for documentation. The

photographs from the 4 groups were randomly arranged and a blind assessment was done to see if the groups could be sorted by the phenotype. Thus, the eyes from the four groups of animals were randomly arranged and were shown one after another, while the observers who were blinded as to the group from which the eyes were derived, categorized each fetus as 'open' or 'closed' eyes. In other words, transparent or opaque eyelids. This procedure was repeated for 9-WT, 10-KO, 8-UCBSC-chimera and 4-hiPSC-chimera. An eye index was assigned to each fetus based on the percentage of the blinded evaluators that deemed the eyelid in the 'open' state. The overall averages of eye lid index was compared between each of the four groups. Statistical differences in eyelid indices were determined using ANOVA and post hoc pair-wise comparison.

4. HEMATOXYLIN AND EOSIN STAINING (H&E)

Once the eyes were sectioned laterally, a sample of 5 slides, with 4 sections on each slide, totaling 20 slices of each eye was stained with H&E to look for structural differences between the 4 groups. The slides were selected in such a way that different areas of the lens would be present for comparison. Thus, one slide at the beginning of the lens (i.e. where the lens just starts to show up), one at the end, and 3 in between were selected for each of the samples. Thus, the 20 slices of eyes that were stained represented all the facets of the eye. Since our main concern is the development of the lens, only those sections consisting of lens tissue were selected. The progressive staining system was used, where the tissues on the slides were first rehydrated with 100 to 50% ethanol washes 2min each and then, stained with hematoxylin pH 4 for 4min. Excess hematoxylin, was then removed by acid washes, by dipping the slides in HCL in 70% ethanol and ammonia in 70% ethanol, 1 and 5 times respectively. The slides were then rinsed under running tap water for about

40 sec. The counter stain Eosin pH 4, was then applied for about 1.5 to 2 min and rinsed under running tap water for 40 seconds, followed by 30 sec in 90% ethanol to remove excess eosin. This was then followed by acid washes with 70% ethanol to 100% ethanol. The slides were then air dried, and the cover slip was applied using U-kit and the slides were sealed.

5. IMAGING

SLIDE SCAN: The sample slides were then scanned using a TISSUEscope LE slide scanner made by Huron Technologies at the University imaging center (UIC), at University of Minnesota. The images were analyzed using macroscan a software also made by Huron Technologies. The size of these images is so large that they can only be opened using Huron viewer, after which they were down sized and were split apart using Fiji/ Image J.

CONFOCAL IMAGING: Immunostained slides were imaged using Nikon NiE C2 Upright Confocal microscope at the UIC, University of Minnesota. A series of z-stack images were obtained, and were compressed to depict the highest fluorescent intensity.

Slides used for IHC to detect HNA positive cells, were imaged using a Lieca fluorescent microscope at the University of Minnesota.

6. IMMUNOHISTOCHEMISTRY

IHC was performed on human-porcine chimeric eyes using an antibody against Human Nuclear Antigen (HNA) to identify human cells. The slides containing the tissues were washed with Phosphate buffered saline (PBS) of pH 7.4 containing 0.1% Tween 20, for

about 5min each in coplin glass jars, repeated thrice. The J-Block (PBS-T + 1% Bovine serum albumin {BSA}) with 1% Tween 20 added to it before use, was used to permeabilize the tissue for 10min. The slides were then incubated for 1 hour in normal donkey serum diluted in PBS containing 10% triton (TX-100) and BSA, to prevent non-specific binding of the primary antibody. HNA primary antibody (1:400, Rockland antibodies, species: mouse polyclonal) was diluted with normal donkey serum in PBS-TX-100-BSA. Tissue sections were incubated overnight at 4°C in primary antibody. The subsequent day the sections were washed with PBS-TX-100-BSA, 3 times/ 5 minutes each, in coplin jars, after which they were incubated for 2 hours in either a donkey anti-mouse 555 Alexa Fluor secondary antibody (1:1000, Invitrogen), diluted in PBS-TX-100-BSA. The slides were washed again in PBS-TX-100-BSA, 4 times/ 5 minutes each in coplin jars. DAPI a nuclear localization stain, used at concentration of 1:1000 and made in PBS was applied for 5 minutes. The slides were then subjected to 3 final washes using PBS-TX-100-BSA, 5 minutes each, after which they were air dried and mounted. Immu-mount was used to preserve the samples and fix on the cover slip and the slides were sealed with a clear nail polish.

Retinal Markers

A) Antibodies to arrestin bind to rod photoreceptors of the retina. The slides containing the tissues were washed with Phosphate buffered saline (PBS) of pH 7.4 containing 0.1% tween 20, for about 5min each in coplin glass jars, repeated twice. The J-Block (PBS-T + 1% BSA) with 1% Tween 20 added to it before use, was used to permeabilize the tissue for 15min. The slides were incubated for 1 hour 30 minutes in normal donkey serum diluted

in PBS containing BSA and 10% triton (TX-100), to prevent non-specific binding of the primary antibody. The sections were incubated with anti-arrestin antibody (1:400, EnCor Biotechnology Inc., species: mouse) and incubated overnight at 4°C. The subsequent day the sections were washed with PBS-TX-100-BSA, 4 times for about 2 minutes, using wash bottles. A goat anti-mouse 488 Alexa Fluor (1:500, Cell Signaling), diluted in (NGS) normal goat serum in PBS-TX-100-BSA, was used as the secondary antibody. The washes were repeated in the same way about 5 times/ 2 minutes each, followed by DAPI for 5 minutes and 3 final washes, 2 minutes each. The slides were then air dried and mounted, in the manner described above.

B) Antibodies against AP2alpha recognize amacrine cells and cells in the ganglion cell layer of the retina (Guduric-Fuchs et al., 2009). Antigen retrieval was performed on the sections using sodium citrate buffer, pH 6, containing 10mM of sodium citrate in 0.05% Tween 20, and incubated in a steamer for 30 minutes, prior to staining. The staining procedure was same as the one mentioned above. The slides were incubated with anti-AP2alpha (1:50, Developmental Studies Hybridoma Bank, species: mouse) and diluted using normal goat serum with PBS-TX-100-BSA. A goat anti-mouse Alexa Fluor 488 (1:500, Cell Signaling) diluted in PBS-TX-100-BSA was used as the secondary antibody.

C) Antibodies against Islet1-1A9 recognize horizontal, ganglion, bipolar and amacrine cells in the retina (Guduric-Fuchs et al., 2009). Antigen retrieval was performed using the previously mentioned, sodium citrate buffer and incubated in a steamer for 30 minutes, prior to staining using the IHC protocol previously mentioned. The Islet1 primary antibody (1:50, Developmental Studies Hybridoma Bank, species: mouse) was diluted to a working

concentration using normal goat serum in PBS-TX-100-BSA. Goat anti-mouse Alexa Fluor 488 (1:500, Cell Signaling) was used as the secondary.

D) To label astrocytes, antibodies against Glial fibrillary acidic protein (GFAP). Staining protocol were the same as above. The slides were labeled with anti-GFAP antibody (1:500, DAKO, species: rabbit) was diluted using normal goat serum in PBS-TX-100-BSA. The secondary antibody used was a goat anti-rabbit Alexa Fluor (1:500, Life Technologies).

RESULTS

3.1 PHENOTYPIC ANALYSIS OF EYES

Once the porcine-human chimeric embryos were harvested after 62 days in gestation, they were excised and organs were harvested. The porcine embryos were documented as shown in figure 9, before they could be perfused and organs were dissected. From the literature we have gleaned that aphakia mice exhibit a closed eyelid phenotype (Rieger et al., 2001). Thus, our first step was to evaluate if such an eyelid phenotype could be recapitulated in a porcine model of *Pitx3*. A photograph depicting the eye of each porcine embryo, wild type pig (WT), *Pitx3* KO pig (KO), *Pitx3* KO injected with hiPSCs (KO+hiPSC) and *Pitx3* KO injected with hUCBSCs (KO+hUCBSCs) were presented to lab members in a random order. The assessment was blind, and the pictures were numbered 1 through 37 and presented in random order. Each individual was required to look at the picture and note if the eyelid was transparent (representing an open eye) or if the eyelid was opaque (representing a closed eye).

FIGURE 9: Representative pictures of porcine embryos used during the phenotypic analysis. **A:** Represents a WT porcine embryo. **B:** KO porcine embryo **C:** KO+hiPSC chimeric embryo **D:** KO+hUCBSC chimeric embryo. As, represented above the pictures were not named and presented in a random order, in order for the assessment to remain impartial.

Pig No.	Pig group	Type	Assesment
1	P3E13	WT	Open
2	P4E2	KO	Closed
3	P3E5	WT	Open
4	P2E6	UCB	Open
5	P1E4	iPSC	Closed
6	P1E3	iPSC	Closed
7	P4E1	KO	Closed
8	P3E2	WT	Open
9	P2E1	UCB	Open
10	P4E5	KO	Closed
11	P4E8	KO	Closed
12	P3E3	WT	Open
13	P2E2	UCB	Closed
14	P5E1	KO	Closed
15	P1E1	iPSC	Closed
16	P4E7	KO	Closed
17	P3E1	WT	Open
18	P2E3	UCB	Intermediate
19	P3E7	WT	Open
20	P2E5	UCB	Closed
21	P5E2	KO	Intermediate
22	P3E17	WT	Open
23	P5E3	KO	Closed
24	P2E4	UCB	Intermediate
25	P3E9	WT	Open
26	P2E8	UCB	Intermediate
27	P1E2	iPSC	Open
28	P3E6	WT	Open
29	P4E4	KO	Closed
30	P5E4	KO	Closed
31	P2E7	UCB	Open
32	P3E8	WT	Open
33	P3E12	WT	Open
34	P4E3	KO	Closed
35	P5E5	KO	Closed
36	P3E10	WT	Open
37	P4E6	KO	Closed

FIGURE 10: Represents the list of eyes presented and the resulting averages of blind assessments**.**

The assessment of each lab member was collected and quantified, as shown in figure 10. Based on the result of the assessment, a graphical representation was created using ANOVA a statistical analysis tool, that provides the 'open eye-index' for chimeric eyes, depicted in figure 11. Each embryo is represented as an individual point. The WT and KO eyes provided the positive and negative controls, respectively. Each group WT, KO,

KO+hUCBSC and KO+hiPSC, are represented with a different color. The assessment from each individual for each embryo was taken and an average was calculated. The eye index was 10 percent if all individuals said open, 90 percent if one individual said closed and everyone else stated open, and so on. The ‘open eye index’ was calculated for each, and from the graph it can be noted that most of the WT eyelids had an ‘open eye index’ of 100%, while the KO had the least, with many falling at 0%. The KO+hUBSC porcine embryos had the greatest distribution, while many fell in the range of the KOs, one in particular had a high index and placed in the WT range. The KO+hiPSCs, had a lower distribution than those embryos injected with UCBSCs. They placed lower and did not have eye indices greater than 50 percent, were closer to the KOs. Thus, from the following analysis we conclude that those embryos injected with human cells, had some improvement in eyelid development. Although, many fell closer to the KO range, there were few among the KO+hUCBSC group that fell in the WT range. Thus, based on the phenotype chimeric embryos P2E1, P2E3, P2E4, P2E6, P2E8 and P1E2 were concluded to have a better development of the eyelid, possibly due to the presence of human cells.

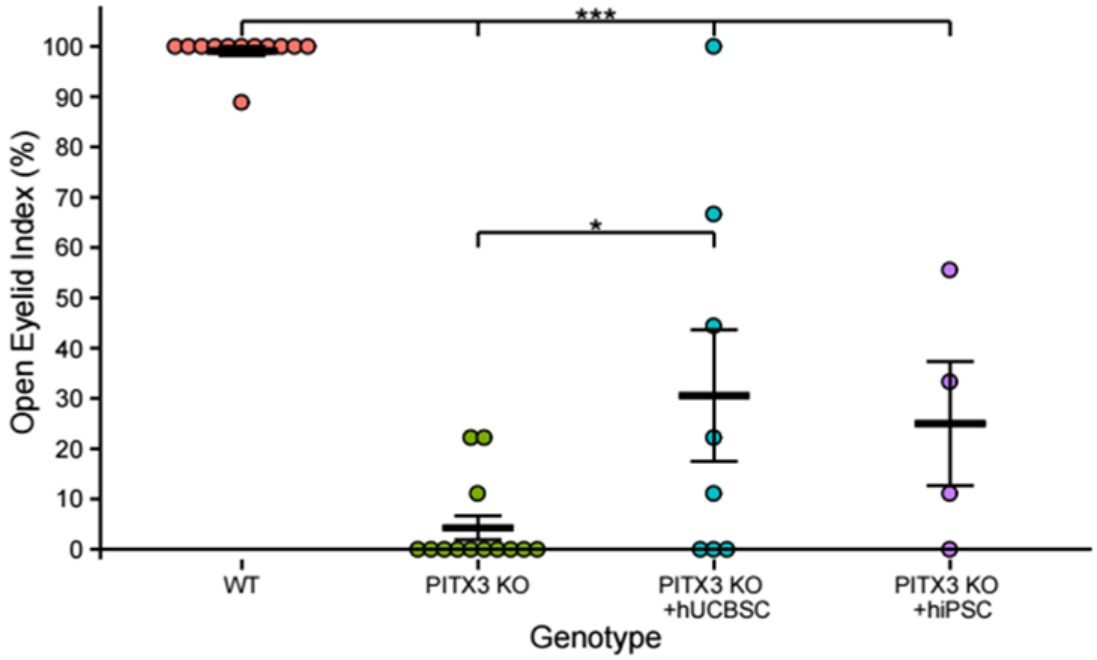

FIGURE 11: Graphical representation of the 'open eye index'. RED: WT porcine eye-lids have a very high 'open eye index', with maximum falling at 100%. GREEN: KO porcine eye-lids have a very low 'open eye index' with most falling at 0%. BLUE: KO+ hUCBSC chimeric porcine eye-lids show a wide distribution, with most falling in the KO range, while a few falling in the WT range. PURPLE: KO+hiPSC chimeric porcine eye-lids show a distribution of eye indices, but fall in the KO range, with none showing greater 60% 'open eye index'.

3.2 ANALYSIS OF EYE HISTOLOGY VIA H&E STAINING

Once the eyes were analyzed for the phenotype, they were embedded in OCT and frozen and stored in -80°C. The tissue blocks were cryosectioned at 8micro meters onto slides for further analyses. The next step was to characterize the morphology of the eyes. According to studies done in *Pitx3* KO mice, a lack of lens has been reported (Rieger et al., 2001; Semina et al., 1998; Varnum and Stevens, 1968). Based on our phenotypic analysis, a few chimeric eyelids were seen to have an improvement compared to the KO, thus we proceeded to see if the structural morphology of those eyes matched the phenotype. It was

also our goal to investigate if the KOs lacked lenses, while the chimeras presented with lenses due to the presence of human cells which provided the expression of *PITX3* gene for lens development. The eye sections were thus stained for Hematoxylin and Eosin (H&E), to visualize and analyze the structural morphology of the eyes. Hematoxylin stains nuclei purple, while the counter stain Eosin stains the lens pink. The right eye from each embryo was used for staining. 5 slides were picked from each embryo, one at the beginning, middle and end of the lens and two in between. Figures 12 through 15, represent the largest (middle) section of eyes, for each embryo, from each of the four groups. It can be noted that the retina stains purple, while the lens stains pink since it lacks nuclei.

Nomenclature: P3E12 is Pig 3, embryo 12. Similarly, P2E2 is pig 2, embryo 2 and so on.

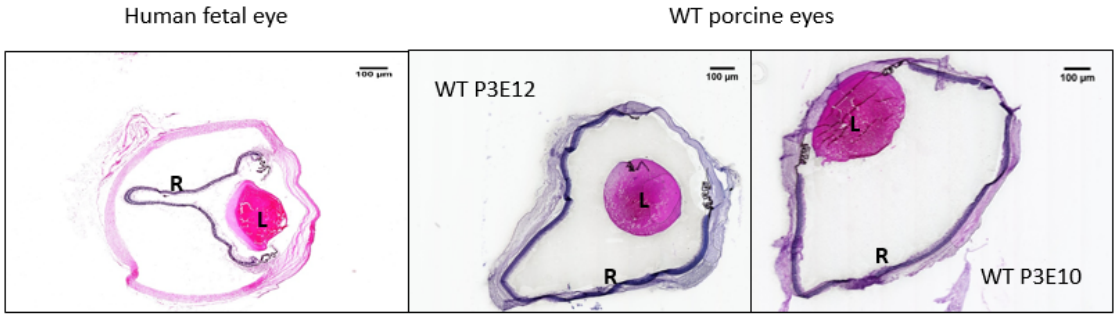

FIGURE 12: H&E stains of human fetal eye about 21 weeks old (**A**) and 62 day old WT porcine eyes (**B** &**C**). **B:** P3E12 **C:** P3E10. **R** depicts the purple retina. While the **L** represents the pink whole lens. These were used as a positive control, to depict how the histology of a normal human and porcine eye looks like. Scale = 100µm.

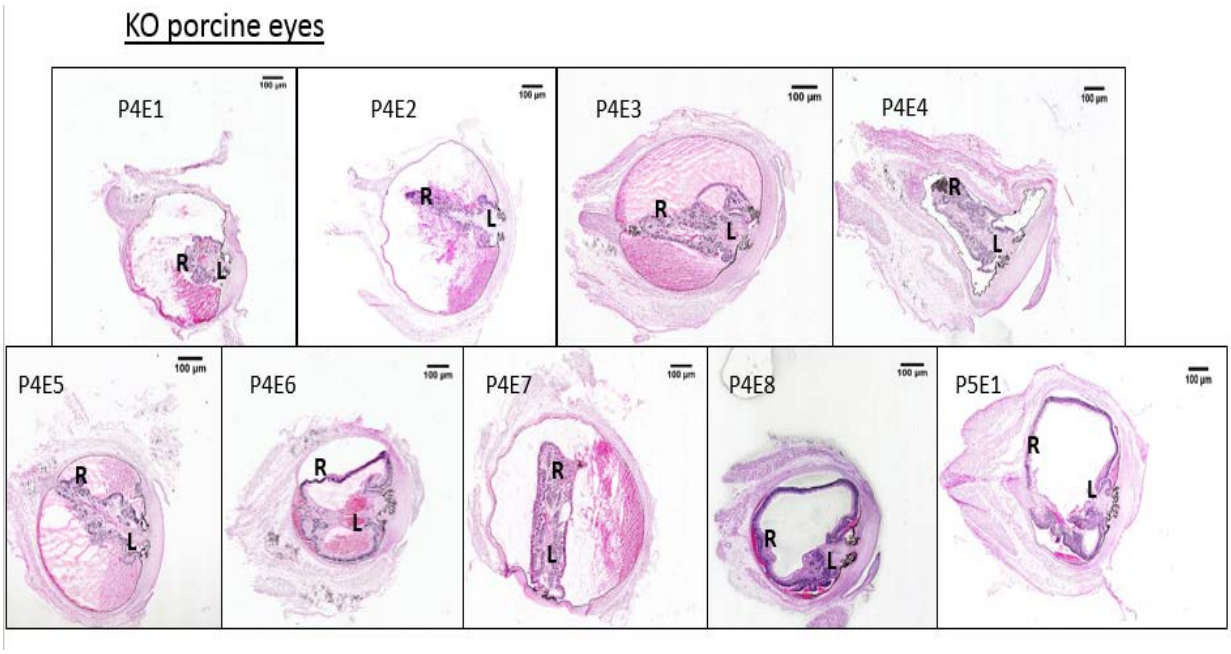

FIGURE 13: KO porcine eyes stained for H&E. **D-K:** are all of the KO used for this study, starting from P4E1 through to E8, while **L:** P5E1. **R** depicts the purple retina. While the **L** represents the lens, in the structure that henceforth will be referred to as 'lens-like structure'. Scale = 100µm.

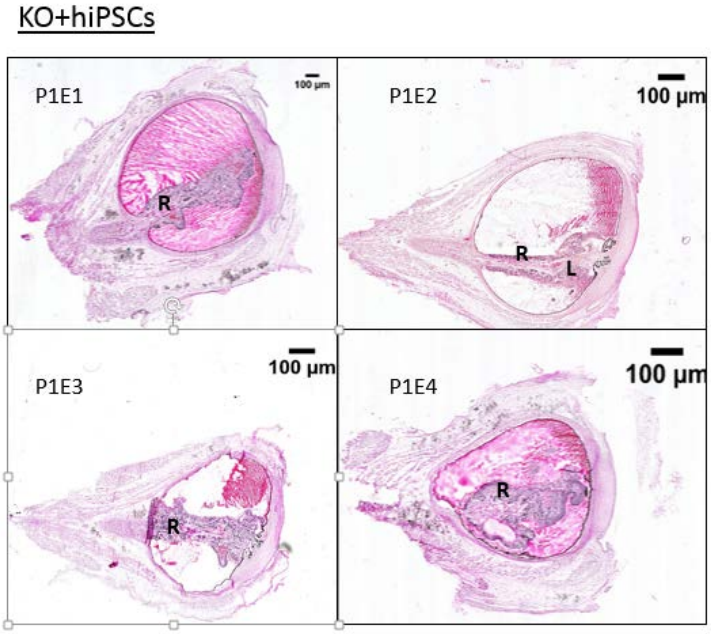

FIGURE 14: H&E stains of hiPSC injected chimeric embryos. **M-P:** are P1E1 to E4 respectively. **R** depicts the purple retina. While the **L** represents the lens, within the 'lens-like structure'. Scale = 100µm.

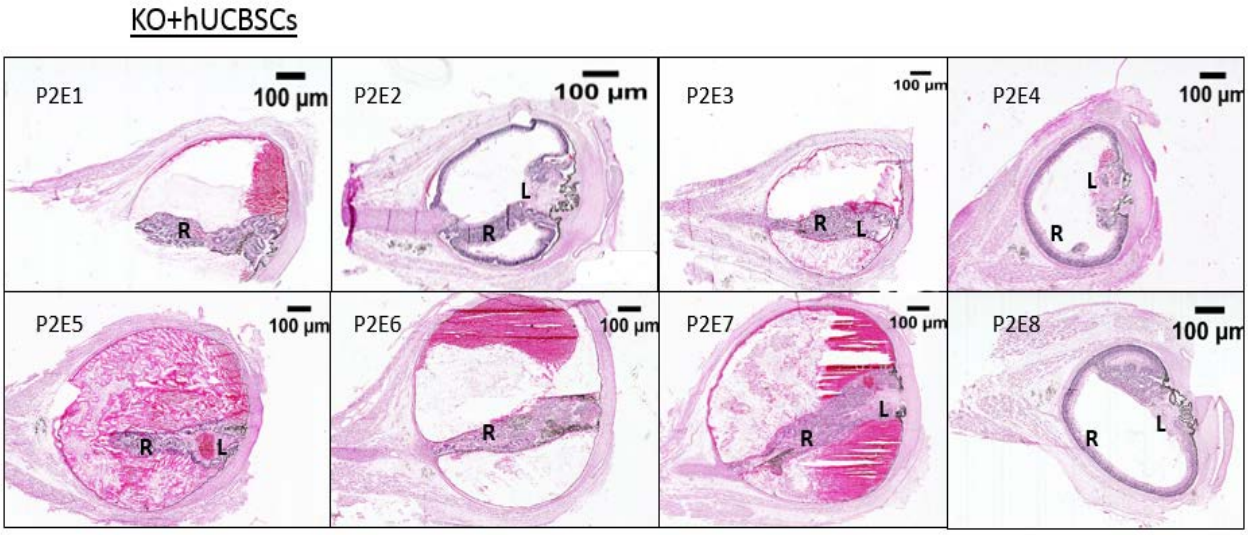

FIGURE 15: hUCBSC injected chimeric eye sections, stained with H&E. **Q-R:** hUCBSC chimeras, P2E1 through E8 respectively. **R** depicts the purple retina. While the **L** represents the lens, within the 'lens-like structure'. Scale = 100µm.

The clump of cells at the center of the eye that stained pink and was in conjunction with the cornea, with purple retinal cells mingled, was designated as 'lens-like structure'. Most of the KO presented with a thin outer layer of retinal pigment epithelial layer (RPE), while most of the retinal cells were clumped together with the lens in 'lens-like structure' that stains mostly purple but pink in certain areas, as depicted in the above figures. They lacked the thick retinal lamination that can be noticed in the WT and human eyes. The chimeras however, portrayed similar morphology as the KOs and little difference was seen between them and the KOs, even though they were complemented with human cells. But, a few of the hUCBSC+KO chimeras, did present with a thick retinal lamination along with the 'lens-like structure', in R, T and X of figure 15. Thus, perhaps this illustrates a certain

improvement, possibly due to the presence of human cells. In saying so, this retinal lamination can be noticed in a few of the KOs as well, seen in I, K and L in figure 13. Thus, in order to confirm that the changes in the hUCBSC+KO chimeras may be due to the action of hUCBSCs, we decided to stains the eyes for human nuclear antigen to detect these human cells.

3.3 IMMUNOHISTOCHEMISTRY FOR THE DETECTION OF HUMAN CELLS

Since, a few of the chimeras presented with a good retinal lamination along with the 'lens-like structure', our hypothesis was that the complementation with human cells may have ameliorated the KO phenotype by providing the expression of *PITX3* gene for lens development. Thus, we decided to perform IHC to the sections of all the chimeric eyes for human nuclear antigen (HNA). WT porcine eyes and the KO were used as negative control as portrayed in figures 17 and 18, while human fetal eyes were used for positive control shown below in figure 16. The sections were stained with an antibody against HNA according to the protocol mentioned in the methods section, shown in figures 19 and 20.

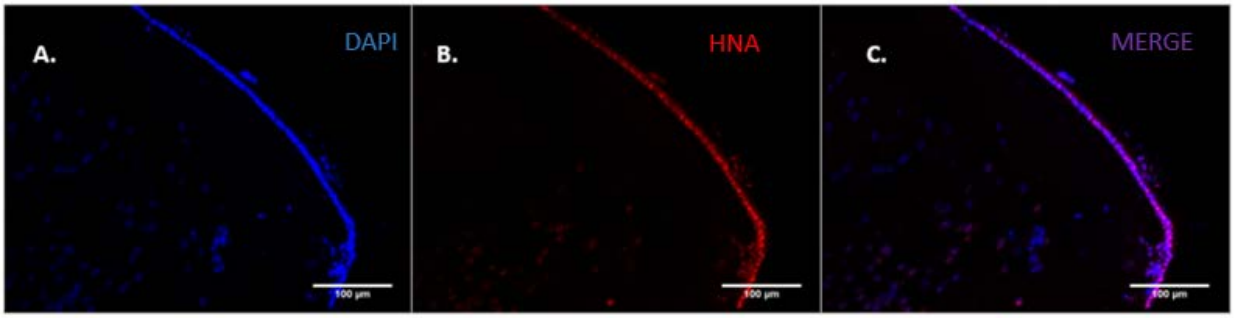

FIGURE 16: IHC of Human fetal eye labeled with anti-HNA antibody. Depicts the epithelial cell layer at the anterior part of the lens. Objective = 20X; Scale: 100µm. **A:** DAPI = Blue. **B:** HNA= Red. **C:** Merge = Purple.

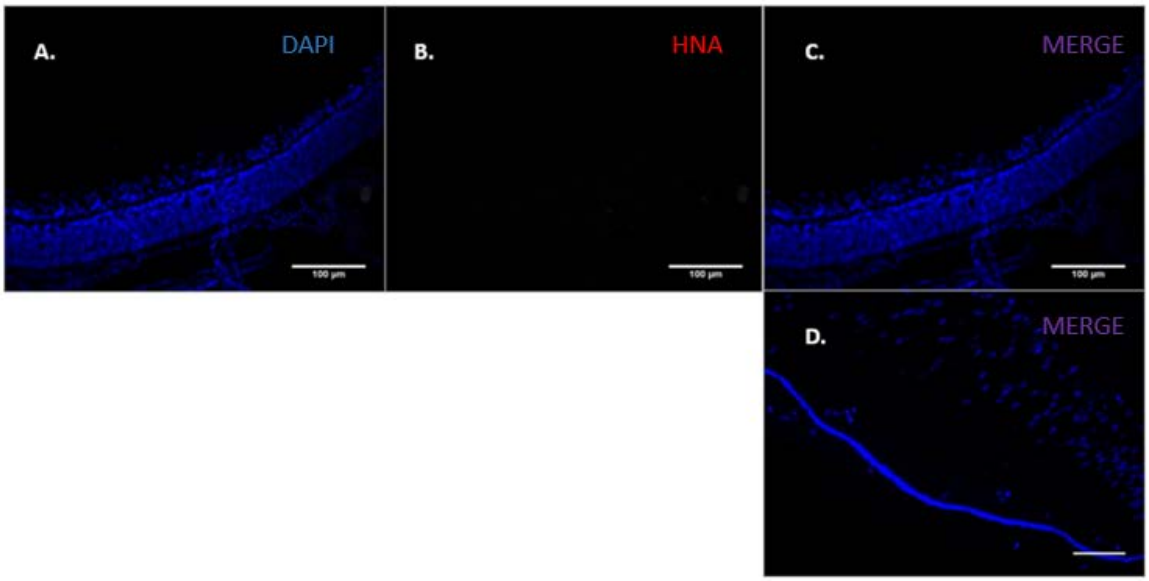

FIGURE 17: IHC of WT porcine eyes labeled with anti-HNA antibody. Depicts WT retina labeled with DAPI. Objective = 10X; Scale: 100µm. **A:** DAPI = Blue. **B:** HNA= Red. **C:** Merge = Purple. **D:** A merged 20X magnification of the epithelial layer at the anterior region of the lens. Scale: 100µm.

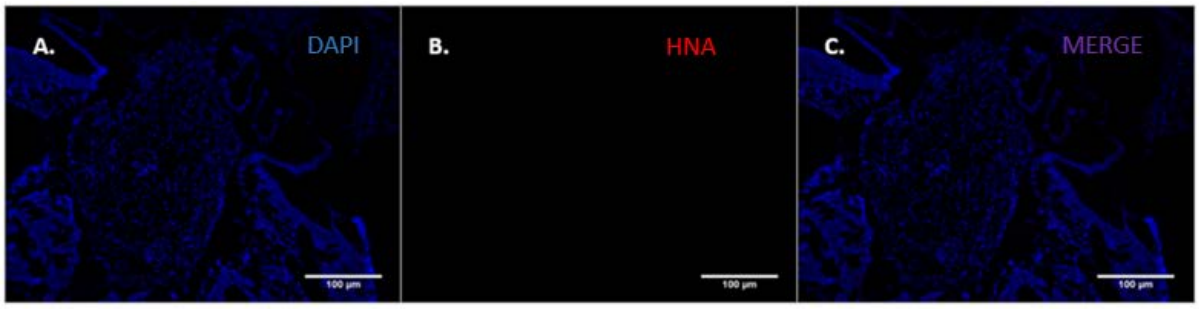

FIGURE 18: KO porcine eyes stained with anti-HNA antibody. The image represents the 'lens-like structure'. Objective = 10X; Scale: 100µm. **A:** DAPI = Blue. **B:** HNA= Red. **C:** Merge = Purple.

FIGURE 19: KO+hUCBSCs chimeric eyes. The image represents the 'lens-like structure'. Objective = 10X; Scale: 100µm. **A:** DAPI = Blue. **B:** HNA= Red. **C:** Merge = Purple.

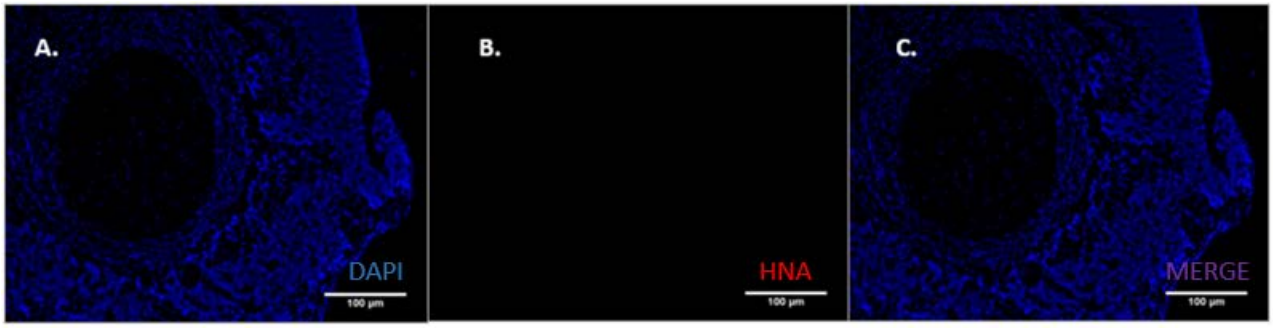

FIGURE 20: KO+hiPSCs chimeric eyes. The image represents the 'lens-like structure'. Objective = 10X; Scale: 100µm. **A:** DAPI = Blue. **B:** HNA= Red. **C:** Merge = Purple.

HNA staining did not dtetect any human cells in the chimeras, and our hypothesis that the subtle changes in retinal pattern between chimeras and KOs possibly due to the presence of human cells, could not be confirmed. The human cells may have been present but were unable to integrate into host, since the action of *Pitx3* is quite late in the developemnt of the eye to provide a niche. Along with *Pitx3, FoxE3* and *Prox1* are also involved in lens differentiation, and may be the reason why an absence of lens was not observed, and their compensation did not provide a wide enough opening for the human cells to integrate and contribute to the development of the organism's eyes.

3.4 IMMUNOHISTOCHEMISTRY TO DETECT RETINAL LAMINATION

The analysis of the changes in retinal lamination between KOs and chimeras, was the next step. This was done by performing immunohistochemistry using antibodies against different retinal cell types such as rods, ganglion cells, mamacrine cells, astrocytes etc, depicted in figures 21-24 below. The goal was to stain for diffterent cell types, visualize their lamination patterns and possibly find a difference between KOs and chimeras. Immunohistochemistry was performed according to the protocol mentioned in the methods. WT porcine cells were used as a positive control as a model of retinal lamination. To label rod photoreceptors in the retina, an antibody against visual arrestin was used to stain eye sections. Panels depicting WT porcine eyes, show clear arrestin-labeled rod photoreceptors near the retinal pigment epithelial layer (RPE), as shown in pannel **A-D** of figure 21. While no proper lamination pattern can be observed in the KOs, **E-H,** figure 21 a clump of retinal cells in the 'lens-like structure' can be observed. Unfortunately, complementation with human cells did nothing to improve retinal lamination as seen in the arrestin staining in KO+hiPSCs, represented in pannels **I-L** of figure 21. However, the lamination for P2E2 KO+hUCBSCs seems to be similar to the WT, **M-P,** figure 21. This can be corroborated by the histological images, which depicted that P2E2 KO+hUCBSCs had a thick, well formed retina (pannel R, figure 15).

Similarly, Glial fibrilary acidic protein (GFAP), stains astrocytes present in the ganglion cell layer (GCL) of the retina(figure 22). AP2 alpha, stains cells in the GCL and amacrine cells in inner plexiform layer (IPL), as portrayed in figure 23. Islet1 antibody labels horizontal, amacrine, bipolar and ganglion cells of the retina. Layers of cells in the GCL, IPL and INL (inner nuclear layer) can be observed in figure 24. A lamination pattern,

similar to the lamination pattern for arrestin was observed for all the above mentioned antibodies.

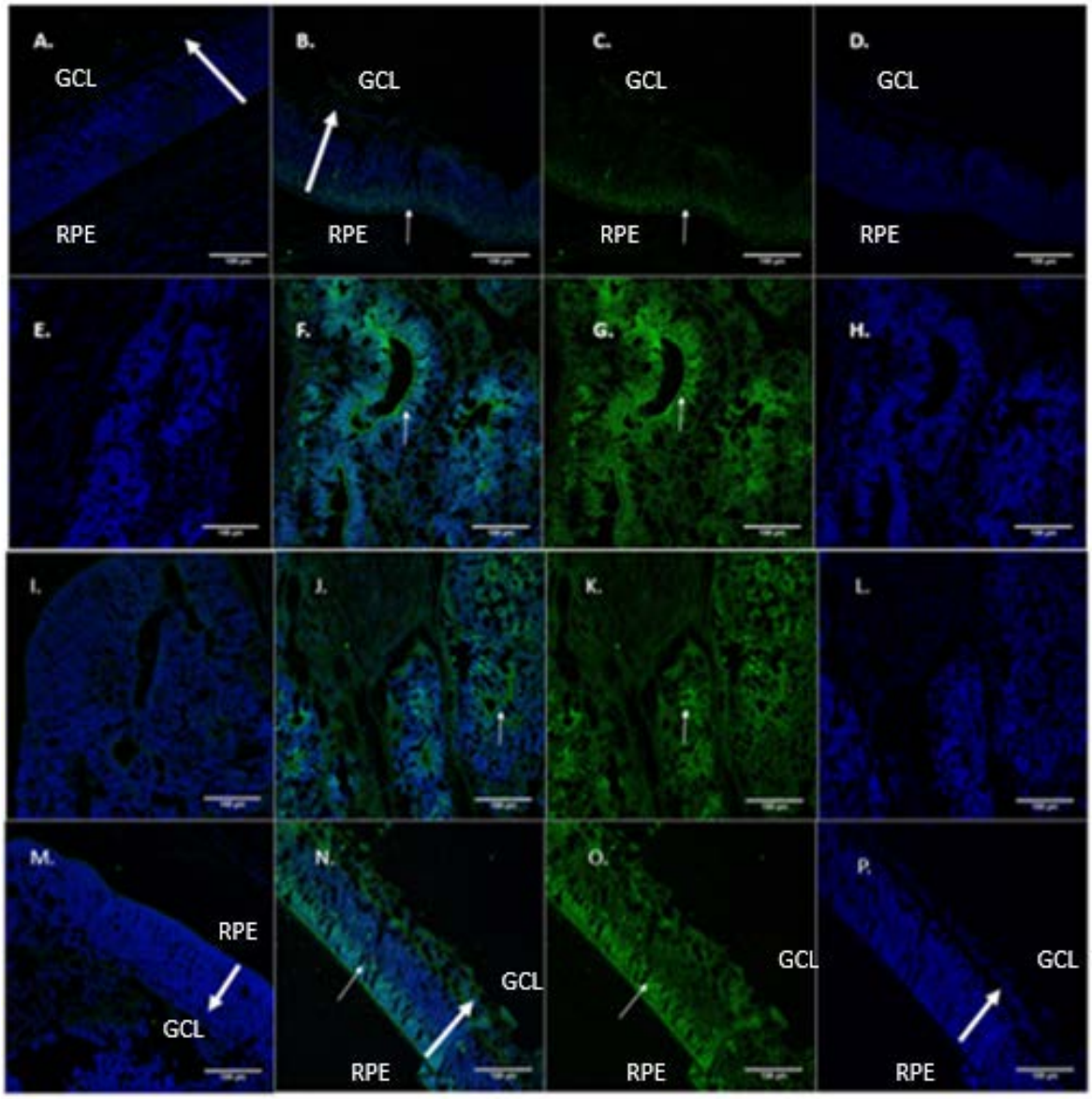

FIGURE 21: IHC for Arrestin on porcine and chimeric eyes. Arrestin labels rod photoreceptors in the retina, that are present right after the RPE. **A-D:** WT P3E12 porcine eyes. **E-H:** KO P4E2 porcine eyes. **I-L:** KO+hiPSCs chimeric porcine eyes. **M-P:** KO+hUCBSC chimeric eyes. **A:** merged image of control, **B:** Merge, **C:** Arrestin = Green and **D:** DAPI = Blue. The thin arrows depict arrestin positive rod photoreceptors. The thick arrows represent retinal orientation. The RPE is present at the base of the arrow, while the GCL is towards the head of the arrow. Objective: 20X. Scale: 100µm.

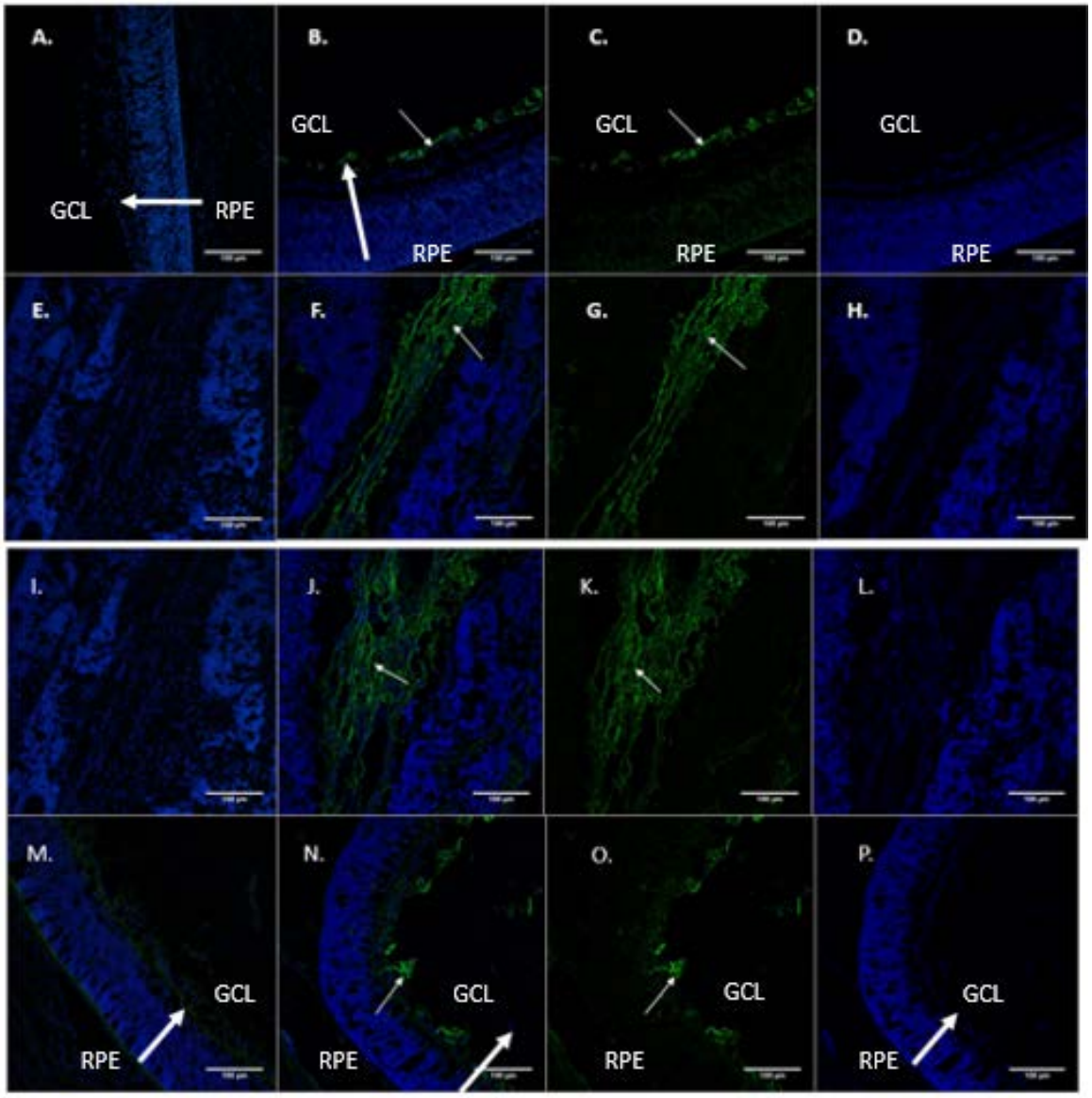

FIGURE 22: IHC for GFAP on porcine and chimeric eyes. GFAP labels astrocytes in the GCL in the retina. **A-D:** WT P3E12 porcine eyes. **E-H:** KO P4E2 porcine eyes. **I-L:** KO+hiPSCs P1E2 chimeric porcine eyes. **M-P:** KO+hUCBSC P2E2 chimeric eyes. **A:** merged image of control, **B:** Merge, **C:** GFAP = Green and **D:** DAPI = Blue. The thin arrows depict GFAP positive astrocytes. The thick arrows represent retinal orientation. The RPE is present at the base of the arrow, while the GCL is towards the head of the arrow. Objective: 20X. Scale: 100µm.

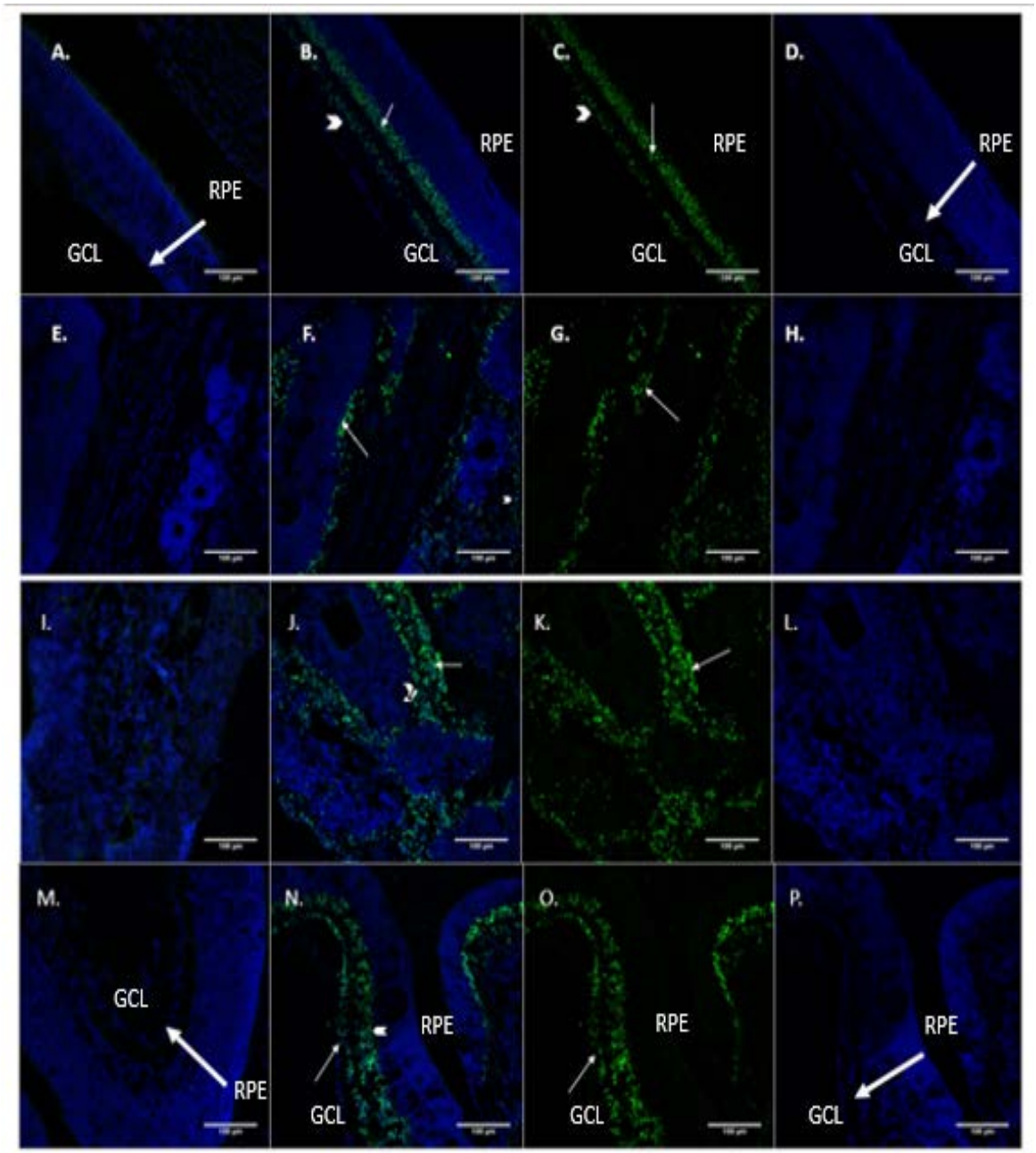

FIGURE 23: IHC for AP2alpha on porcine and chimeric eyes. AP2alpha labels cells in the GCL and amacrine cells in the IPL, in the retina. **A-D:** WT P3E12 porcine eyes. **E-H:** KO P4E2 porcine eyes. **I-L:** KO+hiPSCs P1E2 chimeric porcine eyes. **M-P:** KO+hUCBSC P2E2 chimeric eyes. **A:** merged image of control, **B:** Merge, **C:** AP2alpha = Green and **D:** DAPI = Blue. The thin arrows depict AP2alpha positive cells in the GCL, while the arrow heads indicate the amacrine cells in the IPL. The thick arrows represent retinal orientation. The RPE is present at the base of the arrow, while the GCL is towards the head of the arrow. Objective: 20X. Scale: 100µm.

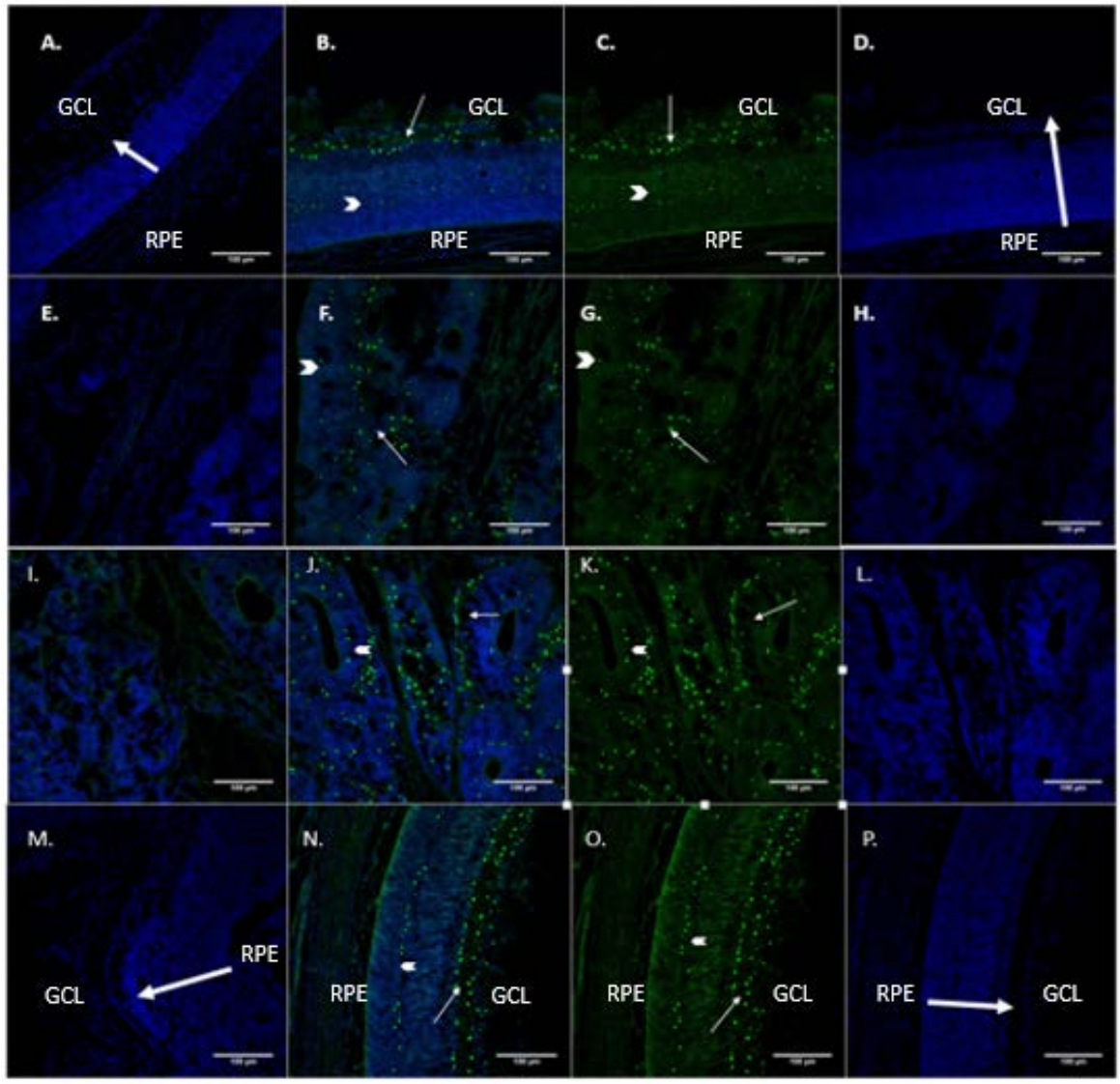

FIGURE 24: IHC for Islet1 on porcine and chimeric eyes. Islet1 labels cells in the GCL, IPL and INL, such as horizontal, amacrine, bipolar and ganglionic cells of the retina. **A-D:** WT P3E12 porcine eyes. **E-H:** KO P4E2 porcine eyes. **I-L:** KO+hiPSCs P1E2 chimeric porcine eyes. **M-P:** KO+hUCBSC P2E2 chimeric eyes. **A:** merged image of control, **B:** Merge, **C:**Islet1 = Green and **D:** DAPI = Blue. The thin arrows depict Islet1 positive cells in the GCL and IPL, while the arrow heads indicate Islet1 positive cells in the INL, such as bipolar and horizontal. The thick arrows represent retinal orientation. The RPE is present at the base of the arrow, while the GCL is towards the head of the arrow. Objective: 20X. Scale: 100μm.

Thus, immunohistochemistry using antibodies against specific retinal cells, permitted the visualization of different cells in the different retinal layers. This has provided proof that retinal lamination exists in all the porcine eyes, including the KOs. This, also confirms the hypothesis that the purple cells in the 'lens-like structure' from the H&E data are retinal cells, and that the retina has failed to disassociate from the lens and has folded together with the lens, due to lack of *Pitx3* expression. Although, no difference in lamination was found, some papers have reported that loss of retinal cells in *Pitx3* KO animals (Shi et al., 2005). Thus, the next step would be to ascertain a qualitative if not a quantitative difference between the four groups.

DISCUSSION AND FUTURE DIRECTIONS

The main purpose of this study was to demonstrate the possibility of using blastocyst complementation as a technique to generate human cells and organs in organogenesis-disabled animal models. Previous studies have indicated that donor cell derived organs have been created in organogenesis disabled animals by genetically manipulating a gene of interest. Complementation studies have thus far been attempted in mice, rats and pigs (Chen et al., 1993; Kobayashi et al., 2010; Liégeois et al., 1996; Matsunari et al., 2013; Usui et al., 2012). The use of human cells for complementation with porcine blastocysts has not been previously reported. In the current study, the possibility of human lens generation using *Pitx3* KO blastocysts, by complementation with hiPSCs and hUCBSCs, has been investigated.

Over the years, many studies have implicated *Pitx3* in the involvement of lens differentiation (Semina et al., 1997, 1998, 2000), not just in mice and rats, but also in zebrafish and xenopus (Khosrowshahian et al., 2005; Shi et al., 2005). *Pitx3* is also involved in SN DA neuron development in the mesDA system (Hwang et al., 2003; Maxwell et al., 2005; Nunes et al., 2003; Smidt et al., 2004a, 2004b), and its connection to Parkinson's disease make it an important gene to study (Le et al., 2011). Many transplantation studies have been evaluated, including the transplantation of human embryonic dopaminergic neurons into patients with PD (Björklund and Lindvall, 2000). However, recovery was reported to be incomplete. Studies have also been done to improve the function of these dopaminergic neurons after transplantation, by treatment with taurourdeoxycholic acid with transplantation studies performed in rats (Duan et al., 2002).

Thus, a way to produce donor-derived organs for transplantation in the central nervous system is important.

Previously, our lab has used human pluripotent stem cells, to confirm that a complementation study using porcine blastocysts is possible (Swaminathan P. 2014). The human pluripotent stem cells were injected into porcine parthenogenic morulae and chimeric embryos were generated, where human cells were observed in the ICM of the blastocysts as well as in liver tissue after 32 days in gestation. Based on that study, *Pitx3* KO blastocysts were generated, and some of these KO blastocysts received hiPSCs and hUCBSCs that were transferred to a surrogate, thus chimeric embryos were generated, by Carlson et.al at Recombinetics.

The phenotypic analysis of porcine and human-porcine chimeric eyes provided us with an 'open eye index'. The WT had an index of 100% since nothing was lacking, and as the positive control the eyelid phenotype was expected to be normal. The KO however, lacking *Pitx3* function, placed at 0% and was the negative control. Thus, by calculating the eye index for the chimeras, our aim was to find out where they placed. If they had a higher index and placed in the range of the WTs, they were deemed to have an improvement. According to our analysis, chimeric embryos P2E1, P2E3, P2E4, P2E6, P2E8 and P1E2 were deemed to have a slightly better phenotype than the KOs.

Thus, next we attempted to corroborate the phenotypic result with morphological analysis, by staining tissue sections with H&E. These sections provided proof of the presence of a 'lens-like structure' in the center of the eye and unfortunately, our study did not recapitulate the lack of lens phenotype seen in aphakia mice (Semina et al., 2000). The WT sections all presented with a thick purple retina, while most of the KOs and chimeras had a thin retina,

with purple cells mixed with the pink lens cells. Some KO+hUCBSC chimeras such as, P2E2, P2E6 and P2E8 had a thick laminated retina. Thus, the phenotypic analysis may have corroborated for these three, and possibility of improvement in eye development due to the presence of human cells was considered. Thus, all eyes were stained against HNA, to detect human cells. Unfortunately, no human cells were detected in any of the eyes. *Pitx3* gene expression may be too late in eye development for the human cells to have a niche to integrate into the host and contribute to lens formation. Perhaps, other genes such as *FoxE3* and *Prox1* have provided enough expression for lens development that the human cells were not provided with an opportunity to contribute (Mochizuki and Masai, 2014; Ogino et al., 2012). The thick retinal lamination was also detected in a few of the KOs as well such as, P4E6, P4E8 and P5E1. Since the KOs were produced from a single clone, this difference in morphology remains unexplained.

Another factor that was noticed from H&E stains were the purple cells in the 'lens-like structure'. No study has previously reported the clumping of the retina, thus to characterize the cells, they were stained with antibodies against retinal cells. Immunohistochemistry, provided proof that they were in-fact retinal cells, mixed in with the lens. On close analysis, it can be noted that some areas of the retina (especially in P2E2 and P2E8) have good structural lamination, while others do not. Since the retina and lens develop from the head ectoderm and later separate out into lens placode and optic cup (Ogino et al., 2012), and we observe a lack of separation in the KOs and chimeras, we report that *Pitx3* may be highly involved in the regulation of this separation and providing us with the possibility of another function for *Pitx3* in eye development. Studies have also reported the loss of certain types of retinal cells in *Pitx3* KO animals (Khosrowshahian et al., 2005). Thus, the next

step is to analyze the images of all sections labeled with retinal markers. The possibility of a qualitative or a quantitative difference existing between the four groups, and the possibility of recapitulating it in porcine models is currently under investigation.

From our experiments we desired to provide evidence that connected the ‘closed/opaque’ and ‘open/transparent’ eyelid phenotype with the lens morphology, which in turn connected the retinal phenotype. We hoped to see that an eye with opaque eyelid, exhibited a lack of lens phenotype or irregular lens formation, as well as degradation of retinal lamination. We also hoped to determine that, complementation with human pluripotent cells had ameliorated the KO phenotype, by rescuing the lens development, or the retinal lamination. However, a clean cut path connecting each of them, was not visible from our studies (refer table figure 25). Our results suggest that *Pitx3* expression may be too late in development to provide an ideal niche for human pluripotent cell integration, was reached. Another point of consideration are the hiPSCs, where the pluripotent cells being injected may not be ‘naïve’ enough to efficiently contribute to the complementation. Although, our results do not provide a positive outcome of the lens, some human cells have been detected in our study of the DA system. The table below shows a summary of these results. Thus, the study has laid the ground work for future experiments. It is important to understand blastocyst complementation in pigs further, by performing complementation studies using porcine stem cells, before moving forward with complementation with human stem cells, which is the next approach of our future experiments, along with investigating the possibility of manipulating transcription factors that are expressed much earlier in development such as, *Lmx1b* along with *Pitx3*.

	EYES				SUBSTANTIA NIGRA	
Fetal Groups	Normal Lens	Thick Retina	Open Eyelid	Human DNA (+)	TH (+) staining	HuNu (+) IHC
hiPSC						
P1E1	(-)	-	-	-	+	-
P1E2	(-)	-	+	-	-	-
P1E3	(-)	-	-	-	-	-
P1E4	(-)	-	-	-	-	-
hUCBSC						
P2E1	(-)	-	+	-	-	-
P2E2	(-)	+	-	-	+/-	-
P2E3	(-)	-	+	-	+/-	-
P2E4	(-)	+	+	-	-	1+
P2E5	(-)	-	-	-	-	-
P2E6	(-)	-	+	-	+	-
P2E7	(-)	-	+	-	-	-
P2E8	(-)	+	-	-	+	1+

FIGURE 25: A summary of results. '+' indicates presence, while '-'indicates absence. While HNA positive cells were observed in the SN none were seen in the lens, with only P2E4 indicating a positive for retina, eyelid and human cells in the SN, while the rest do not show the same level of correlation.

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